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Title: DISORDERS OF SYNAPTIC VESICLE FUSION MACHINERY

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Abbreviations – αSNAP - alpha-soluble NSF attachment protein; AD-ANCL - autosomal dominant adult-onset neuronal ceroid lipofuscinosis; BoNT - botulinum neurotoxins; CSPα – cysteine string protein alpha; DEE - developmental and epileptic encephalopathies; KO - knockout; NSF – N-

ethylmaleimide-sensitive factor; PKD - paroxysmal kinesigenic dyskinesia; PRRT2 - proline-rich transmembrane protein 2; SNAP-25 - synaptosomal-associated protein-25; SNARE - soluble N-ethylmaleimide-sensitive factor attachment protein receptor; STXBP1 - syntaxin-binding protein-1 (Munc18); STXBP5L - syntaxin-binding protein-5-like (tomosyn-2); t-SNARE – target membrane SNARE; v-SNARE – vesicular SNARE; TeNT - tetanus neurotoxin; WT – wild-type; VAMP; vesicle-associated membrane protein.

ABSTRACT

The revolution in genetic technology has ushered in a new age for our understanding of the underlying causes of neurodevelopmental, neuromuscular and neurodegenerative disorders, revealing that the presynaptic machinery governing synaptic vesicle fusion is compromised in many of these neurological disorders. This builds upon decades of research showing that disturbance to neurotransmitter release via toxins can cause acute neurological dysfunction. In this review, we focus on disorders of synaptic vesicle fusion caused either by toxic insult to the presynapse or alterations to genes encoding the key proteins that control and regulate fusion: the SNARE proteins (synaptobrevin, syntaxin-1 and SNAP-25), Munc18, Munc13, synaptotagmin, complexin, CSPα, α-synuclein, PRRT2 and tomosyn. We discuss the roles of these proteins and the cellular and molecular mechanisms underpinning neurological deficits in these disorders.

1. Introduction

Neuronal communication relies on the tightly controlled fusion of synaptic vesicles at nerve terminals, which results in the release of neurotransmitters with strict temporal and quantal precision. At rest, synaptic vesicle fusion is inhibited (Brunger *et al.* 2018). Action potential-mediated opening of voltage-gated Ca^{2+} channels results in Ca^{2+} influx into the nerve terminal, creating a Ca^{2+} nanodomain that triggers the fusion of vesicles that are docked and primed at the plasma membrane, thereby evoking fast synchronous neurotransmitter release (Chanaday & Kavalali 2018, Sudhof 2013). Vesicles can additionally fuse asynchronously following evoked synchronous release and some spontaneous fusion of vesicles also occurs, though this is clamped (Chanaday & Kavalali 2018). These processes are under exquisite regulatory control, preventing excessive neurotransmitter release and ensuring high-fidelity neuronal communication (Rizo 2018, Rizo & Xu 2015, Brunger *et al.* 2019); perturbation of this regulation can lead to a breakdown in neurotransmission.

A range of presynaptic proteins are required to orchestrate these precision events. The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, composed of the vesicular

SNARE protein (v-SNARE) synaptobrevin and the target membrane SNARE proteins (t-SNAREs) syntaxin-1 and SNAP-25 (synaptosomal-associated protein-25), is the core fusion machinery that provides the energy required for fusion of synaptic vesicles with the plasma membrane (Weber *et al.* 1998). Several other key components regulate synaptic vesicle exocytosis in physiological environments (Brunger et al. 2019, Sudhof 2014). SNARE complex assembly is orchestrated by Munc13 and Munc18, and further coordinated by the SNARE chaperones CSPα and α-synuclein. Fusion is facilitated by complexin, rendered Ca^{2+}-dependent by synaptotagmin, and constrained by the accessory proteins PRRT2 and tomosyn.

Given this complexity, it is unsurprising that defects in this machinery result in a range of neurological disorders. Synaptic vesicle fusion as a disease target was first recognised over three decades ago, when it was discovered that some neurotoxins hijack neurotransmitter release by blocking fusion or circumventing the normal tight control of this process (Schiavo *et al.* 1992a, Schiavo *et al.* 1993, Blasi *et al.* 1993, Finkelstein *et al.* 1976, Longenecker *et al.* 1970, Clark *et al.* 1970, Sudhof 2014, Ovsepian *et al.* 2019). Now, new advances in genetic technology are driving a renaissance in our understanding of genetic disorders of synaptic vesicle fusion. This review will focus on how genetic and toxic disruption of the protein machinery that governs synaptic vesicle fusion can give rise to neurodevelopmental, neuromuscular and neurodegenerative disorders (see Table 1). We discuss the roles of the synaptic vesicle fusion proteins implicated in the pathogenesis of these disorders and the potential cellular and molecular mechanisms underpinning neurological deficits in these conditions.

Although Ca^{2+} channelopathies could arguably be considered disorders of fusion, they are outside the scope of this review and have been covered in depth elsewhere (Zamponi *et al.* 2015). Additionally, while there are a plethora of studies indirectly associating presynaptic proteins and genes with susceptibility to neurological and psychiatric disorders, we explore only direct genetic evidence (i.e. likely pathogenic mutations) implicating synaptic vesicle fusion proteins in the aetiology of disease. Contrasting the nuanced perturbation of fusion observed in genetic disorders, toxins will be discussed where relevant to illustrate how acute neurological dysfunction is induced through complete blockade of fusion or changes to Ca^{2+} influx at the nerve terminal.

2. The SNARE proteins

Studies using synthetic lipid bilayer vesicles incorporating v-SNARE synaptobrevin-2 or t-SNAREs syntaxin-1 and SNAP-25 demonstrate that assembly of these proteins constitute the minimum essential machinery for membrane fusion to occur (Weber et al. 1998). However, it should be noted that these proteoliposomal studies represent simplified systems and may not always translate to the physiological environment. The crucial role that SNARE proteins play in synaptic vesicle fusion is best revealed by examining neuronal systems lacking these proteins. Knockout of the primary presynaptic v-SNAREs or t-SNAREs in murine central synapses results in dramatic reductions in action-potential evoked and spontaneous release, as well a large decrease in sucrose-elicited release (which is thought to fuse vesicles that are morphologically docked at the plasma membrane and comprise the "readily releasable pool" of vesicles (Rosenmund & Stevens 1996); but see also (Kaeser & Regehr 2017)) (Schoch *et al.* 2001, Deak *et al.* 2006, Zimmermann *et al.* 2014, Bronk *et al.* 2007, Vardar *et al.* 2016). Cleavage of the SNARE proteins similarly causes a cessation of neurotransmitter release at synapses (Llinas *et al.* 1994, Pumplin & Reese 1977, Mellanby & Thompson 1972). Furthermore, deletion of the SNARE complex assembly factors also abolishes neurotransmitter release (Verhage *et al.* 2000, Augustin *et al.* 1999b).

At a structural level, the SNARE motifs of each protein interact, forming a coiled-coil that zippers into a parallel 4-helix bundle, with SNAP-25 contributing two SNARE helices, and syntaxin and synaptobrevin each contributing one (Sutton *et al.* 1998). At the core of the complex, SNARE proteins interact at 15 periodic "layers" of hydrophobic amino acids and a central layer of ionic interactions (ionic zero layer) (Sutton et *al. 199*8, Fasshauer et al. 1998). The trans-SNARE complex (where v-SNAREs and t-SNARES reside in opposing membranes) forms prior to fusion. Vesicles docked at the plasma membrane are assumed to have at least partially zippered trans-SNARE complexes (Sorensen *et al.* 2006); knockout of any of the three key presynaptic SNARE proteins results in a decrease in docked vesicles (Imig *et al.* 2014).

The trans-SNARE complex is highly dynamic and reversible (Bao *et al.* 2018), and can be disassembled by NSF (N-ethylmaleimide-sensitive factor) and αSNAP (alpha-soluble NSF attachment protein), which also play essential roles in disassembly of SNARE complexes following fusion when they are in the plasma membrane (cis-SNARE complex). Protection against trans-SNARE complex disassembly is provided by several proteins (Prinslow *et al.* 2019), as will be discussed later in this review. Complexin-1 and synaptotagmin-1 bind to the SNARE complex at two sites, known as the primary and tripartite interfaces, to maintain vesicles in a primed and "locked" state (Zhou *et al.* 2017, Zhou *et al.* 2015) with fusion clamped.

Then, together with Ca^{2+} binding and membrane penetration by synaptotagmin-1, the full zippering of the SNARE complex provides the energy required for fusion of the vesicular membrane with the plasma membrane, formation of the fusion pore, and thus neurotransmitter release (Rizo 2018). The transmembrane domains of synaptobrevin and syntaxin-1 and the membrane tethering region of SNAP-25 are postulated to play a key role in the creation of a fusion pore (Sharma & Lindau 2018, Shaaban *et al.* 2019). Following fusion, the SNARE proteins are locked in the cis-SNARE complex on the plasma membrane, awaiting disassembly by NSF and αSNAP for recycling and reuse.

Below, we outline how disruption of fusion can occur through direct impairment of the SNARE proteins, either via toxic action or genetic mutations, and how this leads to neurological dysfunction.

3. Clostridial neurotoxins disrupt fusion by cleaving SNARE proteins

Research into the clostridial neurotoxins tetanus neurotoxin (TeNT, produced by *Clostridium tetani*) and botulinum neurotoxins (BoNT, produced by several *Clostridium* bacteria) formed the foundation of our current understanding of synaptic vesicle fusion. In part, it was the discovery that these toxins act by cleaving the SNARE proteins that revealed the role of these proteins as the essential fusogenic machinery (Schiavo *et al.* 1994, Sudhof *et al.* 1993, Sudhof 2014). Thus, poisoning by clostridial toxins could be considered the first recognised synaptopathies caused by dysfunction of the fusion machinery.

The clostridial neurotoxins are composed of a heavy chain, responsible for uptake of the toxin into neurons, and a light chain, which is a metalloprotease capable of cleaving the SNARE proteins in a highly specific manner (Fig 1). Poisoning with tetanus toxin causes tetanus, which is characterised by progressive tetanic muscle spasms leading to spastic paralysis, due to reduced inhibition of motor neurons. Autonomic dysregulation, due to blockade of release from sympathetic adrenergic neurons, also occurs (Yen & Thwaites 2019). In contrast, botulism presents as flaccid paralysis with early signs including impaired vision, followed by paralysis of facial muscles, and ultimately can result in death from respiratory failure caused by diaphragm paralysis unless mechanically ventilated (Pirazzini *et al.* 2017).

Interestingly, TeNT and BoNT/B target the same sites on the v-SNARE, synaptobrevin. The difference in presentation between tetanus and botulinum poisoning results from the uptake and cellular preference of the toxins. Botulinum toxins are endocytosed into motor neuron nerve terminals and acidification of the endosomal compartment drives translocation of the light chain across the

membrane, which then cleaves SNARE proteins in peripheral cholinergic nerve terminals (Binz & R*ummel 2*009, Rummel 2013, Matteoli et al. *1996,* Colasante et al. *2013,* Harper et al. 2011, Pellett *et al.* 2015). In contrast to BoNT, the majority of TeNT is targeted to non-acidified endosomal compartments following endocytosis at the neuromuscular junction, and then undergoes retrograde trafficking along the axon (Bohnert & Schiavo 2005) before being taken up by upstream neurons (Bomba-Warczak *et al.* 2016) (for review see (Surana *et al.* 2018)). The light chain of TeNT is released into the cytoplasm of inhibitory interneurons of the central nervous system where it cleaves synaptobrevin.

The light chain of TeNT cleaves synaptobrevins, resulting in a blockade of vesicle fusion at nerve terminals (Schiavo *et al.* 1992b, Schiavo et al. 1992a, McMahon *et al.* 1993, Llinas et al. 1994). The light chain of BoNT cleaves the different SNARE proteins at highly restricted sites, dependent on the subtype of toxin (Pirazzini et al. 2017). BoNT/A and E cleave SNAP-25, BoNT/B, D, D/C, F, FA/F_5 and G cleave synaptobrevin, and finally, BoNT/C and C/D cleave both syntaxin and SNAP-25 (Pirazzini et al. 2017) (see Fig 1). With the exception of BoNT/A, this truncates the proteins in such a manner that precludes *de novo* stable SNARE complex formation resulting in a cessation of fusion, although proteins already sequestered in SNARE complexes are comparatively protected (Hayashi *et al.* 1994).

BoNT/A, however, cleaves 9 amino acids from the extreme C-terminus of SNAP-25, well downstream of the core SNARE motifs, maintaining its ability to bind syntaxin-1 and form SNARE complexes (Otto *et al.* 1995, Bajohrs *et al.* 2004). How BoNT/A reduces neurotransmitter release (McMahon *et al.* 1992) remained unknown until it was demonstrated that truncated SNAP-25 inhibits exocytosis in a dominant-negative manner (Huang *et al.* 1998). This truncated protein also increased the flickering of fusion pores (Bao et al. 2018), suggesting that BoNT/A may destabilise the trans-SNARE complex and fusion pore.

Poisoning with botulinum and tetanus toxins represent very severe, potentially fatal, disorders of fusion, brought about by a complete cessation of neurotransmitter release. We will discuss below how genetic advances have now given new insight into the full spectrum of neurological disorders that can be caused by more nuanced perturbation to the presynaptic fusion machinery (Table 1).

4. Synaptobrevins (VAMPs) and their associated genetic disorders

Of the multitude of v-SNARE proteins, only synaptobrevin-1 and synaptobrevin-2 (also called vesicle-associated membrane protein 1 (VAMP1) and VAMP2, together termed synaptobrevins) have been causally linked to neurological disease and will therefore be covered in this review; whereas no

pathogenic variants in non-canonical v-SNAREs (including VAMP3, VAMP4, VAMP7 and Vti1a/b) have been established. Synaptobrevins-1 and -2 have the same domain structure and 77% sequence identity. They possess a less conserved N-terminal proline-rich domain, a single SNARE motif, and are anchored to synaptic vesicles by a C-terminal transmembrane domain, with an extremely short intravesicular tail (Fig. 1) (Archer *et al.* 1990).

4.1 Synaptobrevin-2

Synaptobrevin-2 (encoded by *VAMP2*) is the major synaptic v-SNARE in the central nervous system, where it is highly and widely expressed (Elferink *et al.* 1989, Trimble *et al.* 1990, Raptis *et al.* 2005). In human post-mortem brains synaptobrevin-2 expression is highest in frontal lobes and the putamen (Salpietro *et al.* 2019). In the mouse hippocampus, synaptobrevin-2 is present embryonically then substantially increases expression postnatally (Vuong *et al.* 2018). Rodent studies suggest that synaptobrevin-2 is also expressed in the peripheral nervous system, where it is abundant in autonomic and sensory nerves (Li *et al.* 1996). Synaptobrevin-2 is also found at embryonic and juvenile rodent neuromuscular junctions (Liu *et al.* 2011, Liu *et al.* 2019), but expression wanes in adulthood (Li et al. 1996).

Synaptobrevin-2 knockout (KO) mice die immediately after birth, though display normal brain morphology. Examination of cultured hippocampal neurons revealed an almost complete abolition of action potential-evoked synchronous release, a large reduction in spontaneous fusion events and 10-fold decrease in sucrose-elicited release of the readily-releasable pool (Schoch et al. 2001, Deak et al. 2006, Zimmermann et al. 2014). Additionally, the number of docked synaptic vesicles observed by electron microscopy was dramatically reduced (Imig et al. 2014).

***4.1.1 VAMP2*-associated neurodevelopmental disorder**

Pathogenic mutations in synaptobrevin-2 have only recently been identified in six individuals, giving rise to a neurodevelopmental disorder with autistic features, developmental delay, moderate to severe intellectual disability and infantile hypotonia (OMIM 618760). All variants are *de novo* heterozygous mutations, including four missense mutations (A67P, S75P, F77S, E78A) and two single amino acid deletions (V43del, I45del) (Salpietro et al. 2019, Sunaga *et al.* 2020) (Fig. 1). The two deletion cases were described as less severe, with only moderate intellectual disability, ability to walk, and no movement disorder. In contrast, all four missense cases shared a more severe phenotype, with nonverbal severe intellectual disability, hyperkinetic movement disorders, inability to walk, and

central visual impairment. All patients have disturbed EEG recordings and most experienced seizures, which did not segregate with mutation type (Salpietro et al. 2019, Sunaga et al. 2020).

All six mutations occur in the conserved SNARE motif of synaptobrevin-2, thus perturbation of SNARE complex assembly or function would be the expected mechanism of pathogenicity. However, it is plausible that missense and deletion variants could have distinct pathogenic mechanisms, which may account for varying symptom severity. Whether these mutant proteins express and are properly localised to synaptic vesicles has not been examined, and therefore it is not certain whether these mutations exert dominant-negative effects or cause haploinsufficiency. Haploinsufficiency can be modelled by synaptobrevin-2 heterozygous mice, which have similar viability and longevity to wild-type (WT) animals and no gross behavioural or motor deficits (Schoch et al. 2001, Monteggia *et al.* 2019), but display abnormal anxiety-related behaviour and impaired spatial learning and memory (Koo *et al.* 2015, Orock *et al.* 2019). These mice have impaired synaptic vesicle fusion, decreased basal neurotransmission, increased paired pulse facilitation, reduced long-term potentiation (LTP), and diminished hippocampal glutamate release *in vivo* (Orock et *al. 20*19, Koo et al. 2015, Matveeva et al. 2012). Synaptobrevin-2 heterozygous mice were found to be resistant to electrical hippocampal seizure kindling (Matveeva et al. 2012), which is at odds with the seizure phenotype seen in some patients with synaptobrevin-2 mutations.

Only two disease variants have been functionally investigated: S75P and E78A, in a proteoliposome fusion assay (Salpietro et al. 2019). For the S75P mutant, the rate of fusion was decreased and Munc18-stimulated fusion was more profoundly impacted. Inclusion of S75P and WT synaptobrevin-2 into liposomes in a 1:1 ratio produced similar effects, indicating that this variant has the capacity to act in a dominant-negative manner (Salpietro *et al.* 2017). A previous study revealed that a S75E,Q76H double mutation did not affect trans-SNARE assembly, but blocked stimulation of trans-SNARE zippering by Munc18 (Shen *et al.* 2015). In cultured mouse cortical neurons where WT synaptobrevin-2 was replaced with S75E,Q76H synaptobrevin-2, the mutant protein localised to nerve terminals but decreased action potential-evoked, spontaneous, and sucrose-elicited release and release probability (Shen et al. 2015). Thus, this variant may cause slowing of Munc18-dependent SNARE zippering and fusion in a dominant-negative manner. No proteoliposome fusion defect was detected for the E78A mutant (Salpietro et al. 2019), indicating that this variant can form SNARE complexes and interact with Munc18 *in vitro*. However, this reduced system doesn't examine many other interacting proteins nor replicate physiological membranes, leaving the specific pathogenic mechanism of the E78A mutation undetermined. The other human variants (V43del, I45del, A67P, and F77S) were not examined in this or any other study.

Missense mutations in synaptobrevin-2 cluster in the C-terminal region of the SNARE motif, which is important for calcium-triggered fusion and late steps in exocytosis (Sor*ensen e*t al. 2002, Wal*ter et* al. 2010, Gil *et al.* 2002). A67 and F77 are situated at core hydrophobic layers of the SNARE complex, known to be sensitive to mutation (Fasshauer et al. 1998). Substitution of proline at A67 could disrupt interactions of this residue with the t-SNAREs in the same layer and may kink the synaptobrevin-2 α-helix and impact coiled-coil SNARE complex formation. The asymmetric packing of F77 against 3 small alanines from SNAP-25 and syntaxin may align and orient the SNARE complex (Fasshauer et al. 1998). Mutation of F77 to alanine nearly eliminated evoked release from chromaffin cells, although did not impact the affinity of synaptobrevin-2 for the t-SNARE complex (Walter et al. 2010). However, the human variant (hydrophilic serine) could more severely disrupt this tight interaction.

The effects of single amino acid deletions V43del and I45del are hard to predict, but could possibly alter the downstream alignment of the periodic hydrophobic layers, disrupting or distorting SNARE complex formation or reducing zippering efficiency. V43 and I45 are found in the N-terminal region of the SNARE motif, which is important for t-SNARE binding and nucleation of the coiled-coil complex, as well as docking and priming, and therefore the readily-releasable pool (Hao *et al.* 1997, Hua & Charlton 1999, Walter et al. 2010, Wiederho*ld et a*l. 2010, Yavuz et al. 2018, Ellena et *al. 20*09, Sorensen *et al.* 2006).

4.2 Synaptobrevin-1

Based on homology, synaptobrevin-1 (encoded by *VAMP1*) has been assumed to perform a similar function to synaptobrevin-2, but in distinct neuronal subsets. Across mammalian species, including humans, synaptobrevin-1 is robustly expressed in the spinal cord as well as found in some brain regions (Elferink et al. 1989, Trimble et al. 1990, Li et al. 1996, Nystuen *et al.* 2007, Liu et al. 2011, Bourassa *et al.* 2012, Shen *et al.* 2017). While the pattern of synaptobrevin-1 expression in the human brain is unknown, in rodents it is highest in somatomotor nuclei of the cerebellum, midbrain and brain stem. The presence of synaptobrevin-1 in the cerebrum is debated and potentially confined to discrete neuronal populations, possibly with preference for inhibitory synapses (Trimble et al. 1990, Raptis et al. 2005, Nystuen et al. 2007, Imig et al. 2014, Zimmermann et al. 2014, Ferecsko *et al.* 2015, Vuong et al. 2018). In the periphery, synaptobrevin-1 is expressed in cranial nerves and motor neurons (Li et al. 1996, Liu et al. 2011, Peng *et al.* 2014). Both synaptobrevin-1 and synaptobrevin-2 are found at embryonic and juvenile mouse neuromuscular junctions, but synaptobrevin-1 becomes predominant by adulthood (Li 1996, Liu 2011, Liu 2019, Peng 2014). Evidence from neurotoxin BoNT/D studies

corroborates that synaptobrevin-1 is also the major VAMP isoform at the human neuromuscular junction (Peng et *al. 201*4, Coffield et *al. 199*7, Eleopra et *al. 201*3, Yamamoto et al. 2012).

There is a paucity of data on the differential splicing of synaptobrevin-1, but at least 3 splice isoforms are expressed in humans. Splicing is thought to be tissue-specific, producing neuronal synaptobrevin-1A and –1D, and non-neuronal mitochondrial synaptobrevin-1B (Isenmann *et al.* 1998, Berglund *et al.* 1999, Bourassa et al. 2012, Shen et al. 2017). These isoforms all differ only by their last exon that encodes the small intravesicular C-terminus (Berglund et al. 1999). Synaptobrevin-1 has been implicated in the aetiology of two distinct disorders.

4.2.1 VAMP1 Congenital Myasthenic Syndrome

Homozygous mutations in synaptobrevin-1 cause a congenital myasthenic syndrome (OMIM 618323). Affected individuals presented with infantile hypotonia and severe muscle weakness. Other reported symptoms varied amongst cases and include moderate to severe motor delay, areflexia, contractures, facial myopathy, and ophthalmic abnormalities. All heterozygous family members are unaffected. Overall 5 homozygous synaptobrevin-1 variants have been reported across 7 individuals, comprising three frame shift mutations (G18Wfs*5, E43Gfs*24 and c.340delAfs), one splice site variant (c.129 + 1G>A) and one missense mutation (R49P) (Monies *et al.* 2017, Shen et al. 2017, Salpietro et al. 2017).

Most variants are likely to result in loss of synaptobrevin-1 function. Two of the three frame shift mutations (G18Wfs*5 and E43Gfs*24) result in truncation of synaptobrevin-1 (Salpietro et al. 2017, Monies et al. 2017), and are likely to undergo nonsense-mediated decay. While some truncated cytosolic protein could still be expressed (Salpietro et al. 2017), these would be missing most, or all, of the SNARE motif. Another mutation (c.129 + 1G>A) affects the 5' splice site between exons 2 and 3 (Monies et al. 2017). The consequence of this mutation on splicing wasn't examined but may cause loss of function through exon skipping or other interruption to the SNARE motif. The single homozygous missense mutation (R49P) (Salpietro et al. 2017) occurs in a residue which, in synaptobrevin-2, is important for trafficking to synaptic vesicles (Koo *et al.* 2011). Moreover, the recessive nature of this disorder suggests that this would be a loss-of-function variant and therefore may have a distinct pathogenic mechanism from the likely dominant-negative, missense mutations in synaptobrevin-2.

The consequences of synaptobrevin-1 deficiency have been examined in synaptobrevin-1 null "lethal wasting" mice (Nystuen et al. 2007). While heterozygous mice were phenotypically normal, null mouse pups lacked purposeful limb movements, curled and clasped limbs, became immobile around 10 days

old and died by 15 days old (Nystuen et al. 2007). Neuromuscular junctions from two-week-old synaptobrevin-1 null mice were morphologically normal, but functionally compromised. Release probability, evoked synchronous release, and spontaneous fusion were all reduced, though the estimated size of the electrically-evoked readily-releasable pool was unaffected. Asynchronous release was also amplified and both Ca^{2+}-sensitivity and Ca^{2+}-cooperativity of release were markedly impaired (Liu et al. 2011). The persistence of some neurotransmission contrasts the near-abolition of neurotransmitter release in synaptobrevin-2 KO hippocampal neurons, indicating that other v-SNAREs are likely to be present at the neuromuscular junction during development.

The final mutation is a frame shift (c.340delAfs) causing extension of the C-terminus by 66 amino acids for synaptobrevin-1A (Ile114Serfs*72) and 29 amino acids for synaptobrevin-1D (Ser114Valfs*34). This case was particularly severe, and the affected individual died of respiratory failure at 14 years old (Shen et al. 2017). Both elongated synaptobrevin-1A and -1D variants were shown to express in HEK293T cells, but at ~15% and ~70% of WT levels, respectively (Shen 2017), suggesting that low levels of elongated synaptobrevin-1 may have been expressed in the affected individual. Expression of elongated synaptobrevin-1 isoforms could not rescue evoked exocytosis in synaptobrevin null chromaffin cells (Shen et al. 2017). It is unclear how erroneous extension of the intravesicular C-terminus would affect protein function; however, the polarity and charge of the intravesicular residues has been shown influence localisation of synaptobrevin-1 and fusion pore expansion (Isenmann et al. 1998, Ngatchou *et al.* 2010).

4.2.2 VAMP1 Spastic Ataxia

A form of hereditary spastic ataxia (SPAX1, OMIM 108600), with onset between 10-20 years of age, has been ascribed to a heterozygous point mutation in synaptobrevin-1 (Grewal *et al.* 2004, Bourassa et al. 2012). For synaptobrevin-1A (and non-neuronal synaptobrevin-1B) this mutation (c.340+2T>G) affects the 5′ splice site between exons 4 and 5, interrupting splicing of these variants and instead yielding a product similar to synaptobrevin-1D. For synaptobrevin-1D, translation normally continues past this splice site and terminates four amino acids downstream. Therefore, this mutation (c.342T>G) instead substitutes the first amino acid (S114R) of the intravesicular C-terminus (Bourassa et al. 2012).

This autosomal dominant disorder was originally thought to be caused by haploinsufficiency of synaptobrevin-1A. However, given that the heterozygous family members of individuals with synaptobrevin-1 congenital myasthenic syndrome are asymptomatic (see previous), a dominant-

negative mechanism better explains pathogenesis of synaptobrevin-1-associated spastic ataxia (Monies et al. 2017, Shen et al. 2017).

The effect of this aberrant splicing on neuronal health or synaptic vesicle fusion has not been investigated and the consequences of this mutation are difficult to predict as little is known about the different functions, or even tissue expression, of synaptobrevin-1 splice isoforms. The divergent intravesicular domains of synaptobrevin-1 isoforms (and the synaptobrevin-1D missense variant) could confer different fusogenic properties, as the C-terminal tail of synaptobrevins has been demonstrated to regulate both fusion pore expansion and the subcellular localisation of the protein (Isenmann et al. 1998, Ngatchou et al. 2010). This emerging clinical relevance encourages further exploration of the distinct synaptobrevin-1 splice isoforms as well as the role of the intravesicular tail of VAMPs in synaptic vesicle fusion. Moreover, the delayed onset of this spastic ataxia suggests that there may be a developmental switch in the expression of synaptobrevin-1 isoforms or that dependence of cerebellar and motor neurons on VAMPs could vary over the human lifespan.

5. SNAP-25

SNAP-25 (encoded by the *SNAP25* gene) is widely distributed throughout the brain (Oyler *et al.* 1992, Boschert *et al.* 1996) and is the major SNAP family member involved in neurotransmitter release. However, SNAP-23 is also expressed in the brain and may contribute to exocytosis, especially post-synaptically (Kadkova *et al.* 2019). SNAP-25 contains a short N-terminus, and two SNARE motifs separated by a long linker region (Fig. 1), and is unique amongst the synaptic SNARE proteins in that it contributes two SNARE motifs to the SNARE complex and lacks a transmembrane domain. Instead, palmitoylation of cysteine residues in the linker region is responsible for tethering SNAP-25 to the plasma membrane (Veit *et al.* 1996), and facilitates fusion triggering and pore expansion (Shaaban et al. 2019). SNAP-25 is localised to presynaptic nerve terminals but also other neuronal membranes including axons and dendrites (Galli *et al.* 1995). There are two splice variants of SNAP-25, SNAP-25a and SNAP-25b, which differ by 9 amino acid substitutions within the C-terminal half of the first SNARE motif and the start of the linker region. A developmental switch in isoforms is essential for survival (Bark *et al.* 2004); SNAP-25a is the major isoform in embryonic mice, but SNAP-25b becomes the dominant isoform in central and peripheral neurons upon weaning (Bark *et al.* 1995, Boschert et al. 1996). Given the essential role played by SNAP-25 as a presynaptic t-SNARE, it is unsurprising that homozygous SNAP-25 KO mice are embryonic lethal (Washbourne *et al.* 2002). While low-density hippocampal cultures from SNAP-25 KO mice degenerate after approximately a week in culture (Washbourne et al. 2002), high-density cultures survive but display decreased spontaneous and

sucrose-stimulated release, and a very large reduction in evoked synchronous release (with 2/3 neurons displaying no response) (Bronk et al. 2007).

5.1 SNAP-25 developmental and epileptic encephalopathy

Seven published heterozygous mutations in SNAP-25 have been found in individuals with neurodevelopmental delay and epilepsy (Heyne *et al.* 2018). A further 10 variants have very recently been identified in a comprehensive phenotypic characterisation of SNAP-25 developmental and epileptic encephalopathy (DEE; preprint (Klöckner *et al.* 2020). Mild to profound intellectual disability was observed in all cases and many presented with other neurological comorbidities. Of the published variants, K40E, G43R, V48F, D166Y, and a truncating mutation (Q174∗) affect both SNAP-25 isoforms (Rohena *et al.* 2013, Hamdan *et al.* 2017). Two mutations have been identified which specifically affect SNAP-25b: R59P, which was associated with only mild developmental delay (Fukuda *et al.* 2018), and I67N (Shen *et al.* 2014).

All identified mutations occur in the SNARE motifs and are predicted to destabilise the SNARE complex or disrupt interactions with key binding partners including synaptotagmin-1 and αSNAP (Zhou et al. 2015, Klöckner et al. 2020), but detailed functional studies are largely lacking. Of the published mutations, only I67N and D166Y have experimental evidence of pathogenicity. The best studied disease variant is SNAP-25b-specific I67N, which lies in a hydrophobic layer, impairs t-SNARE folding, and destabilises the linker and C-terminal domains of the SNARE complex without affecting the N-terminal region. This reduces the total energy released by SNARE complex formation and slows down zippering, selectively compromising the “power stroke” that drives membrane fusion (Rebane *et al.* 2018). This SNAP-25b variant also inhibits fusion of liposomes and causes dominant-negative impairment of exocytosis in chromaffin cells (Shen et al. 2014). “Blind-drunk” mice, which contain an alternative mutation in the same residue (I67T) (Jeans *et al.* 2007), have motor deficits and mild behavioural disturbances. Additionally, there are large impairments in spontaneous and evoked synchronous release, and defects in replenishment of the readily-releasable pool (Jeans et al. 2007).

In contrast, D166Y is likely to impact vesicle priming (Schupp *et al.* 2016). Mutation of D166 to alanine did not compromise SNARE complex stability or release probability; however, it was unable to fully rescue sucrose- or action potential-evoked release when introduced into SNAP-25 null autaptic hippocampal cultures. This variant also potentiates spontaneous release, compared to WT SNAP-25 (Schupp et al. 2016). Only one nonsense mutation (Q174*) has been identified, truncating SNAP-25 midway through the second SNARE motif. This variant may escape nonsense-mediated decay,

allowing it to block fusion in a dominant-negative manner. Truncation of SNAP-25 by BoNT/A results in toxicity despite SNARE motifs remaining intact, highlighting that the C-terminus of SNAP-25 is important for its function.

6. Syntaxin-1

Syntaxin-1 (historically referred to as HPC-1 or P35) is composed of an N-terminal peptide, an alpha-helical domain (the Habc domain), and a C-terminal SNARE motif and transmembrane region (Fig. 1). Outside the SNARE complex, syntaxin-1 adopts a "closed" inactive conformation facilitated by binding to Munc18, whereby the Habc domain folds back and occludes the SNARE motif entering into the trans-SNARE complex (Dulubova *et al.* 1999, Misura *et al.* 2000). Munc13 helps transition syntaxin-1 to an open conformation, allowing it to engage in the ternary SNARE complex (Ma *et al.* 2011).

Whilst there are several syntaxin family members, syntaxin-1A and syntaxin-1B (encoded by *STX1A* and *STX1B* respectively) are the dominant paralogs involved in neurotransmitter release. Syntaxin-1A and B are co-expressed in most brain regions in rodents, albeit at differing levels (Ruiz-Montasell *et al.* 1996), but have differential expression in the peripheral nervous system. Sensory neurons and perivascular nerves are enriched in syntaxin-1A, while motor endplates and muscle spindles predominantly express syntaxin-1B, and both are expressed in the enteric nervous system (Aguado *et al.* 1999). Deletion of both syntaxin-1A and B in mice is embryonic lethal and leads to gross morphological abnormalities and neuronal cell loss both *in vivo* and *in vitro* (Mishima *et al.* 2014, Vardar et al. 2016). Syntaxin-1 is important for neuronal maintenance and survival, but this is dissociable from its essential role in neurotransmission (Vardar et al. 2016). Depletion of both syntaxin-1A/B resulted in a cessation of action potential-evoked, spontaneous and sucrose-elicited release, and a 50% decrease in morphologically docked vesicles (Vardar et al. 2016).

Despite being widely expressed in the CNS, syntaxin-1A is not essential for life; syntaxin-1A KO mice are viable, have no gross pathological features, and are fertile (Fujiwara *et al.* 2006, Gerber *et al.* 2008). These mice display normal spontaneous and evoked release, basal transmission and paired-pulse ratio, but impaired LTP (Fujiwara et al. 2006, Gerber et al. 2008). *STX1A* is one of approximately 28 genes falling within the commonly deleted region in Williams Syndrome (or Williams-Beuren Syndrome, OMIM 194050), a multi-system developmental syndrome with features including cardiovascular disease, connective tissue abnormalities, delayed motor development, mild intellectual disability, unique social and personality characteristics, and growth and endocrine abnormalities (Osborne *et al.* 1997, Nakayama *et al.* 1998, Morris 2017). While *STX1A* is unlikely to be a major causative gene, syntaxin-1A haploinsufficiency could contribute to cognitive features in some cases

(Botta *et al.* 1999, Tassabehji *et al.* 1999, Wu *et al.* 2002, Gao *et al.* 2010). Correspondingly, heterozygous and homozygous syntaxin-1A KO mice show some behavioural abnormalities in social and object recognition, fear conditioning, and acoustic startle response (Fujiwara et al. 2006, Fujiwara *et al.* 2010, Fujiwara *et al.* 2016).

In contrast, syntaxin-1B is the dominant isoform required for neuronal survival and function, as syntaxin-1B KO mice have severely disrupted motor coordination, impaired brain development and die by two weeks of age (Kofuji *et al.* 2014). Syntaxin-1B KO cultured hippocampal neurons were more prone to cell death, and also exhibited impaired vesicle docking and increased paired-pulse ratio, but there was no change in action potential-evoked excitatory or inhibitory release (Mishima et al. 2014, Wu *et al.* 2015). The effects on spontaneous and sucrose-elicited fusion was more variable, and were reduced in one study (Mishima et al. 2014) but unaffected in another (Wu et al. 2015). In contrast to small central synapses, evoked release was impaired at the neuromuscular junction of syntaxin-1B KO mice, together with augmented short term depression (Wu et al. 2015).

6.1 Syntaxin-1B-associated epilepsies

Heterozygous mutations in syntaxin-1B cause a wide spectrum of epileptic disorders from genetic generalised epilepsies (GGE) and genetic epilepsies with febrile seizures plus (GEFS+), to more severe developmental and epileptic encephalopathies (DEE) that feature early-onset intractable seizures, cognitive regression, and neurological deficits (see Wolking *et al.* (2019) for a comprehensive review of the clinical spectrum of *STX1B*-associated epilepsies). At least 26 distinct heterozygous syntaxin-1B mutations have been identified, including 10 missense mutations, 5 nonsense mutations, 5 frameshift mutations, 1 complex insertion-deletion, two splice site variants, and 3 cases of *STX1B* whole gene deletion (Schubert *et al.* 2014, Wolking *et al.* 2019, Vlaska*mp et a*l. 2016, Peres et al. 2018, Krenn *et al.* 2020). It is important to note that seizure type varies even within families harbouring the same mutation and incomplete penetrance was observed in two large families (Schubert et al. 2014, Wolking et al. 2019).

Most nonsense and frameshift mutations truncate the protein before or within the SNARE motif and would be downregulated by nonsense-mediated decay, with one exception of a frameshift (Thr285Aspfs*75) that occurs in the transmembrane domain and would elongate the protein, with unknown effects on variant expression, trafficking and function. Therefore, these variants, in addition to whole gene deletion cases, would all be expected to cause haploinsufficiency of syntaxin-1B. Missense mutations in syntaxin-1B are distributed throughout the protein but concentrated in the

conserved SNARE motif. Intriguingly, there is an early suggestion of a possible genotype-phenotype relationship as 6 of 7 patients or families with missense mutations in the SNARE motif have developmental and epileptic encephalopathy, whereas 5 of 6 patients or families with a comparatively milder condition (genetic epilepsies with febrile seizures plus) possessed truncating variants (Wolking et al. 2019).

Haploinsufficiency of syntaxin-1B as a pathogenic mechanism can be investigated in heterozygous animals. However, syntaxin-1B heterozygous mice have a very mild phenotype, with normal growth, survival, and brain structure, and no effect on action potential-evoked, sucrose-evoked, or spontaneous release at hippocampal or neuromuscular synapses (Wu et al. 2015). This is in contrast to zebrafish larvae with 50% knockdown of syntaxin-1B, which lacked normal touch response and exhibited paroxysmal movements and jerks (Schubert et al. 2014). Epileptiform events were evident in approximately half of these animals, which increased in frequency upon acute elevation in temperature (analogous to the febrile seizures experienced by many affected individuals). Expression of patient variant V216E could not rescue epileptiform activity in knockdown animals, suggesting that this substitution causes loss of syntaxin-1B function (Schubert et al. 2014).

While the pathophysiological impacts of human syntaxin-1B mutations have rarely been investigated, three human variants (a complex indel, V216E and G226R) have recently been examined (Vardar *et al.* 2020). These had differential effects on protein stability, Munc18-1 and Munc13-1 binding, neuronal survival, and neurotransmitter release when introduced into syntaxin-1B KO neurons. However, expression of these variants in syntaxin-1B heterozygous neurons (mimicking the heterozygous nature of *STX1B* epilepsies) had no effect on short-term plasticity or action potential-evoked, sucrose-elicited, or spontaneous release, except the indel variant that decreased spontaneous release (Vardar et al. 2020). These detailed studies highlight that these variants, which are associated with different clinical severity, likely act via distinct pathogenic mechanisms, without inducing gross defects in neurotransmission.

7. SNARE complex assembly factors: Munc18-1 + Munc13-1

Munc18-1 (also known as syntaxin-binding protein-1 (STXBP1)) and Munc13-1 play essential complementary roles in SNARE complex assembly amongst other presynaptic activities. Munc18-1 (encoded by *STXBP1* in humans and comprising domains 1-4) is a member of the Sec1/Munc18 (SM) family of proteins. Munc13-1 is the major neuronal Munc13 isoform in mammals (encoded by *UNC13A* gene in humans) and is highly expressed in the olfactory bulb, striatum, cortex, hippocampus and

cerebellum, although Munc13-2 and Munc13-3 are also expressed in the brain (Augustin *et al.* 1999a). Munc13-1 contains four protein kinase C homology domains (one C1 and three C2), a calmodulin-binding domain (CaMb) and an elongated tethering module known as the MUN domain (Fig. 1).

The importance of Munc18-1 and Munc13-1 for vesicle fusion is perhaps best exemplified by the severe phenotypes of mice lacking either protein. Munc18-1 KO mice have an almost complete abrogation of neurotransmission and die immediately after birth; furthermore, cultured neurons from these mice undergo degeneration (Verhage et al. 2000, Heeroma *et al.* 2004). Munc13-1 KO mice also die a few hours after birth (Augustin et al. 1999b). Hippocampal autaptic cultures from Munc13-1 KO mice have a massive reduction in sucrose-mediated and action potential-evoked glutamatergic release, with no effect on GABAergic release (Augustin et al. 1999b). Munc13-1/-2 double KO neurons have a complete cessation of evoked and spontaneous excitatory and inhibitory neurotransmitter release and are insensitive to application of hypertonic sucrose (Varoqueaux *et al.* 2002). Munc13-1/2 KO mouse synapses and unc18 null *C. elegans* synapses also have a large reduction in docked vesicles (Imig e*t al. 2*014, Siksou et *al. 200*9, Gracheva et al. 2010).

Munc18-1 and Munc13-1 play multi-faceted roles to orchestrate and "template" SNARE complex assembly, ensuring that the SNARE proteins are in the correct orientation (Lai *et al.* 2017). The binary complex formed between Munc18-1 and syntaxin-1 suppresses entry of syntaxin-1 into non-productive and incorrectly oriented SNARE complexes and likely constitutes the starting point for synaptic vesicle fusion (Rizo 2018). Free syntaxin-1 is secured by Munc18-1 into a closed, inactive confirmation that prevents entry into the ternary SNARE complex (Dulu*bova et* al. 1999, Hata et al. 1993) and is important for regulating fusion (Gerber et al. 2008). In order for fusion to proceed, the MUN domain of Munc13-1, together with Munc18-1, opens syntaxin-1 (Ma et al. 2011, Yang *et al.* 2015). Munc13-1 also binds to the membrane-proximal region of synaptobrevin-2; this interaction templates SNARE complex formation, making synaptobrevin-2 more accessible to the Munc18-1/syntaxin-1 complex (Wang *et al.* 2019). Munc18 also binds to synaptobrevin-2 (Sitarska *et al.* 2017). SNAP-25 can then associate to form a half-zippered SNARE complex, releasing syntaxin-1 from Munc18-1 and resulting in full SNARE complex assembly.

The importance of Munc13-1 and Munc18-1 is fully appreciated when considering the dynamic nature of the trans-SNARE complex. Munc13-1 and Munc18-1 enhance assembly and prevent disassembly of trans-SNARE complexes in the presence of NSF-αSNAP (Prinslow et al. 2019), and also prevent de-priming of synaptic vesicles in neurons (He *et al.* 2017). Additionally, Munc13-1 can bridge the synaptic vesicle and plasma membranes, which contributes to docking of synaptic vesicles and is essential for spontaneous and evoked release (Quade *et al.* 2019).

Mutations in Munc18-1 cause *STXBP1*-encephalopathies, which are being covered in a separate review in this special issue by Abramov *et al.* (2020) and will not be discussed in further detail here. Two disease-causing mutations in Munc13-1 have thus far been identified. A homozygous nonsense mutation (Gln102*) in *UNC13A* was identified in a girl with microcephaly and thin corpus callosum, cortical hyperexcitability, marked hypotonia, hyporeflexia, and minimal movement; she died at 4 years from respiratory failure (Engel *et al.* 2016). Electrophysiological assessment of an anconeus muscle specimen from the child revealed severely reduced spontaneous release frequency and deficits in evoked release (Engel et al. 2016). This mutation would likely result in complete loss of Munc13-1 expression; correspondingly, Munc13-1 KO mice display weakness, reduced breathing rate and die hours after birth. Cultures from these mice have severe deficits in evoked and sucrose-mediated release (Augustin et al. 1999b). Loss of Munc13-1 would cause syntaxin-1 to be stuck in the closed position (Engel et al. 2016) and would also affect vesicle docking.

Subsequently, a *de novo* heterozygous missense mutation in Munc13-1 (P814L) was identified in a child with delayed neurological development, intellectual disability, dyskinesia, autistic spectrum disorder and comorbid attention-deficit hyperactivity disorder (ADHD) but with normal EEG and brain structure (Lipstein *et al.* 2017). When introduced into murine Munc13-1/2 KO autaptic cultures, this variant intriguingly increased synaptic vesicle fusogenicity and release probability. Short term plasticity was altered and both evoked synchronous release and spontaneous release were augmented. *C. elegans* expressing the P814L variant on a WT background had greater acetylcholine secretion than worms expressing WT Munc13, indicating a dominant-negative increase in release (Lipstein et al. 2017). Thus, loss- and gain- of-function mutations in Munc13-1 have been shown to cause neurodevelopmental disorders with distinct clinical presentations. Given that Munc13-1 KO mice have deficits only in glutamatergic release (Augustin et al. 1999b), it would be expected that any alterations to neurotransmission resultant from Munc13-1 mutations would predominantly affect excitatory neurons.

8. SNARE chaperones: CSPα and α-synuclein

The critical importance of correctly formed and productive SNARE complexes is also guarded by chaperone proteins that perform quality control of SNARE proteins, prevent aberrant protein interactions and facilitate optimal SNARE complex assembly.

8. 1 Cysteine-string protein alpha (CSPα)

Cysteine-string protein alpha (CSPα) (encoded by *DNAJC5)* acts as a vital co-chaperone of SNAP-25 (Tobab*en et a*l. 2001, Stahl *et al.* 1999, Sharma *et al.* 2011, Gordon & Cousin 2014). While the other mammalian CSP family members are only expressed in the testes (CSPγ), or testes and brain (CSPβ) (Fernández-Chacón *et al.* 2004, Gundersen *et al.* 2010), CSPα is present in most tissues (Chamberlain & Burgoyne 1996), but is highly localised to synaptic vesicles in all neurons (Mastrogiacomo *et al.* 1994). As a member of the Hsp40 family of co-chaperones (also known as DnaJ proteins; reviewed by Gorenberg and Chandra (2017)), CSPα has a J domain that binds to the ATPase Hsc70 (Caplan *et al.* 1993, Braun *et al.* 1996), a C-terminal domain that enhances this interaction (Stahl et al. 1999), and a cysteine-string domain that contains several palmitoylation sites that drive correct targeting of this protein to synaptic vesicles (Greaves & Chamberlain 2006) (Fig. 1).

CSPα forms a trimeric complex with the co-chaperones Hsc70 (heat-shock cognate 70) and SGT (small glutamine-rich tetratricopeptide repeat-containing protein) on the surface of synaptic vesicles (Tobaben et al. 2001). Through modulation of Hsc70-SNAP-25 interactions, CSPα alters the conformation of the t-SNARE to prevent SNAP-25 aggregation and degradation, and to facilitate docking, priming and SNARE-complex assembly (Sharma et al. 2011). CSPα also modulates fusion kinetics and enhances exocytosis by facilitating fusion pore opening in a phosphorylation-dependent manner (Evans *et al.* 2001, Stevens *et al.* 2003, Shiraf*uji et* al. 2018, Chiang *et al.* 2014).

Heterozygous mutations in the gene encoding CSPα cause autosomal dominant adult-onset neuronal ceroid lipofuscinosis (AD-ANCL; also known as Parry disease and autosomal dominant Kufs disease, OMIM 162350). Post-mortem examination of AD-ANCL brains reveal autofluorescent neurolysosomal deposits of lipopigments (Noskova *et al.* 2011). Symptoms of the disease have an average onset of 30 years of age (Burgoyne & Morgan 2015), and include ataxia, myoclonus, seizures, cerebral and cerebellar atrophy, and progressive dementia (Boehme *et al.* 1971, Noskova et al. 2011, Velinov *et al.* 2012, Cadieux-Dion *et al.* 2013). AD-ANCL culminates in premature death, typically within ten years of symptom onset (Boehme et al. 1971, Noskova et al. 2011).

Three heterozygous CSPα mutations (L115R, L116del and C124-C133dup) associated with AD-ANCL have been identified, which all lie within the cysteine-string domain. All variants affect membrane affinity and/or impair palmitoylation, thus resulting in mislocalisation of the protein to the plasma membrane (Benitez *et al.* 2011, Noskova et al. 2011, Jedlickova *et al.* 2020). Interestingly, L115R and L116del mutations also increase the propensity of CSPα to oligomerise with WT CSPα, which reduces Hsc70 ATPase-stimulating activity (Zhang & Chandra 2014). These oligomers are also prone to degradation (Zhang & Chandra 2014), which may explain the reduced levels of CSPα within the brains

of AD-ANCL patients (Noskova et al. 2011). Together, this suggests that loss of normal CSPα function likely underlies AD-ANCL. As such, CSPα KO systems may be appropriate models for this disorder.

Following 2-4 weeks of normal development, CSPα KO mice undergo neurodegeneration, resulting in muscle weakness and impairments in locomotion, culminating in death by three months (Fernández-Chacón et al. 2004). These mice exhibit age-dependent deficits in synaptic transmission at the neuromuscular junction (Fernández-Chacón et al. 2004), where there is also less evoked release, slowed priming rate, heightened synaptic depression, and diminished readily-releasable pool (Rozas *et al.* 2012). SNAP-25 protein expression (but not mRNA expression) is downregulated both in CSPα KO mice and in cell culture systems depleted of CSPα, leading to a decrease in SNARE complex assembly (Chandra *et al.* 2005, Rozas et al. 2012, Sharma et al. 2012a, Sharm*a et al*. 2011). There is also increased proteasomal degradation of SNAP-25 and Hsc70 in CSPα KO brains (Sharma et al. 2011). The impairment in stability and/or assembly of SNARE complexes in CSPα KO mice correlated with the degree of neurodegeneration, suggesting that impairments in this step of fusion directly contribute to the neurodegenerative phenotype (Sharma et al. 2012a, Sharma et al. 2011). Notably, SNAP-25 protein levels were also reduced in human AD-ANCL brains (Benitez *et al.* 2015), providing further evidence that loss of SNAP-25 and decreased SNARE complex assembly contribute to pathogenicity in AD-ANCL.

Intriguingly, overexpression of another presynaptic chaperone protein, α-synuclein, has been shown to ameliorate the neurodegenerative phenotype of CSPα KO mice, while absence of α-synuclein worsens the phenotype (Chandra et al. 2005). Although α-synuclein overexpression does not restore diminished SNAP-25 expression in CSPα KO mice, SNARE complex assembly is rescued, which indicates that α-synuclein acts downstream of CSPα to directly promote SNARE assembly (Chandra et al. 2005, Sharm*a et al*. 2011, Burre et al. 2010). These findings support the idea that discrete impairments in steps of fusion can be the main causative mechanism underpinning some forms of neurodegeneration, and that synaptic dysfunction can precede, and may underlie, synaptic and neuronal loss.

8.2 α-Synuclein

α-Synuclein is a small chaperone protein encoded by the *SNCA* gene (also known as *PARK1*) (Maroteaux & Scheller 1991, Ueda *et al.* 1993). This protein is primarily and highly expressed in neurons, where it is predominantly localised to presynaptic terminals, and is enriched in the olfactory bulb, neocortex, striatum, hippocampus, and notably the substantia nigra (Iwai *et al.* 1995, Li *et al.* 2002, Tagu*chi et* al. 2016, Mori *et al.* 2002). α-Synuclein is one of three members of the synuclein

protein family, which share significant sequence homology, and are at least partially functionally redundant (Jakes *et al.* 1994, Lavedan 1998). Compensation by β- and/or γ-synuclein may thus account for the relatively mild phenotype of α-synuclein KO mice; these mice develop normally although do display changes in synaptic vesicle pool distribution and mobilisation (Cabin *et al.* 2002), and a reduction in striatal dopamine (Abeliovich *et al.* 2000).

α-Synuclein has been studied extensively due to its histopathological and genetic ties to a swathe of progressive neurodegenerative pathologies termed synucleinopathies. Lewy bodies and Lewy neurites, enriched in misfolded aggregated α-synuclein, are the pathological hallmarks of Parkinson's disease and Lewy body dementia (Yoshimoto *et al.* 1995, Giasson *et al.* 2003). In Parkinson's disease, degenerative cell loss spreads from the brainstem and olfactory bulb throughout neighbouring regions of the brain (Braak *et al.* 2003). Death of dopaminergic neurons in the substantia nigra pars compacta and the resulting loss of dopaminergic input into the striatum underlies the motor symptoms of resting tremor, rigidity, bradykinesia and postural instability (Guatteo *et al.* 2017). However, prodromal symptoms, including hyposmia, constipation and depression can occur decades before motor symptoms (Mahlknecht *et al.* 2015). Lewy body dementia patients additionally present with dementia within one year of onset of motor symptoms. Dementia is also common in Parkinson's disease, but with later onset (Orme *et al.* 2018). Multiple system atrophy is characterised by neuronal loss, movement and coordination impairment, and autonomic system degeneration (reviewed by Whittaker *et al.* (2017)). A defining trait of this disorder is the presence of glial cytoplasmic inclusions rich in filamentous α-synuclein (Spillantini *et al.* 1998).

Parkinson's disease can be caused by autosomal dominant duplications, triplications or missense mutations (A30P, E46K, H50Q, G51D, A53E and A53T) in *SNCA* (Polymeropoulos *et al.* 1997, Kruger *et al.* 1998, Zarranz *et al.* 2004, Appel-Cresswell *et al.* 2013, Kiely *et al.* 2013, Proukakis *et al.* 2013, Pasanen *et al.* 2014). Interestingly, there is heterogeneity in clinical phenotype across the different mutations, with variation in age-of-onset, pattern of cell loss and rate of progression amongst other traits (Whittaker et al. 2017). The G51D and A53E mutations and triplication of *SNCA* are associated with a multiple system atrophy-like phenotype (Pasanen e*t al. 2*014, Kiely e*t al. 2*015, Singleton et al. 2003, Fuchs *et al.* 2007), while E46K has been identified in Parkinson's disease and Lewy body dementia (Zarranz et al. 2004). Each of these missense mutations can increase the aggregation of α-synuclein into intracellular inclusions (Chartier-Harlin *et al.* 2004, Ibáñez *et al.* 2004, Burre *et al.* 2012). It remains unknown whether aggregation of α-synuclein induces pathogenesis via direct toxicity or causes loss of synuclein function (reviewed by Benskey *et al.* (2016)); both may contribute to disease. Interestingly, α-synuclein can also co-aggregate *in vitro* with disease-associated variants of Munc18-

1, which is hypothesised to underlie Parkinsonian symptoms observed in some older individuals with *STXBP1*-encephalopathy (Chai *et al.* 2016, Guiberson *et al.* 2018, Abramov et al. 2020).

α-Synuclein is composed of an N-terminal membrane-associated region, and acidic C-terminal tail which binds to the N-terminus of synaptobrevin-2 (Burre e*t al. 2*012, Burre et al. 2010, Diao et al. 2013, Chandra *et al.* 2003) (Fig. 1). The membrane-associated region assumes an α-helical conformation upon binding to membranes such as small synaptic vesicles (Jo et al*. 2000,* Davidson *et al.* 1998, Middleton & Rhoades 2010). The membrane-associated region also contains the non-amyloid-β component (NAC) domain, which has a high propensity to misfold into β-sheets, seeding aggregation of the protein into pathogenic protein species (Serpell *et al.* 2000).

An evolving hypothesis suggests that synaptic dysfunction may underlie early Parkinsonian prodromal symptoms, preceding neuronal loss (reviewed by Bridi and Hirth (2018)). α-Synuclein is proposed to play a role in various stages of the synaptic vesicle cycle including fusion, endocytosis, and vesicle clustering (Nemani *et al.* 2010, Lautenschlager *et al.* 2017, Sulzer & Edwards 2019); disease-related mutations can interfere with each of these functions. With respect to its role as a regulator of fusion, there are several different mechanisms by which α-synuclein may contribute to disease pathogenesis. α-Synuclein binding to synaptobrevin-2 potentiates SNARE complex assembly, in a manner dependent on membrane association (Burre et al. 2014, Burre *et al.* 2010). Aged α/β/γ-synuclein triple KO mice have deficits in SNARE complex assembly and these mice have age-dependent neurological deficits and die prematurely, suggesting that loss of synuclein function may contribute to disease aetiology (Burre et al. 2010, Greten-Harrison *et al.* 2010). Moreover, there is a decrease in SNARE complex assembly in Parkinson's disease brains (Sharma *et al.* 2012b). A30P impairs both membrane binding and SNARE complex assembly, however E46K and A53T did not (Burre et al. 2012). Physiological concentrations of α-synuclein facilitated vesicle docking *in vitro* (again, dependent on binding to synaptobrevin-2 and membranes) while higher concentrations inhibited docking (Lai *et al.* 2014, Lou *et al.* 2017, Hawk *et al.* 2019). While some studies report that α-synuclein has no effect on fusion kinetics (Larsen *et al.* 2006, Lai et al. 2014, Hawk et al. 2019), α-synuclein enhanced exocytosis in chromaffin cells and hippocampal neuronal cultures by dilating the fusion pore and decreasing pore closure. These effects were abolished by A30P and A53T mutations (Logan *et al.* 2017). Therefore α-synuclein may play direct roles in synaptic vesicle fusion, and disease-associated mutations or changes to expression may impact this.

Additionally, α-synuclein can form annular pore-like oligomers in the presence of membranes with sufficient anionic lipid content, thereby increasing membrane permeability either by acting as ionic pores or through membrane "thinning" (Volles & Lansbury 2002, Zakharov *et al.* 2007, Stockl *et al.*

2013, Tosatto *et al.* 2012). Pore-like oligomers composed of A53T, A30P or E46K mutant α-synuclein induce permeabilisation more readily than WT α-synuclein (Volles & Lansbury 2002, Furukawa *et al.* 2006, Tsigelny et al. 2012, Zakharov *et al.* 2007). Both resting intracellular calcium concentration (Furukawa et al. 2006, Tsigelny et al. 2012) and KCl-induced calcium influx was heightened in cell lines expressing A53T or A30P α-synuclein (Furukawa et al. 2006), which may "poison synaptic function" (reviewed by Bezprozvanny and Hiesinger (2013)) via dysregulation of calcium-dependent fusion in a similar manner to toxins that circumvent the fusion machinery (see later). These pore-like oligomers, which have been isolated from glial cytoplasmic inclusions in human multiple system atrophy patients (Pountney *et al.* 2004), may be a contributing factor to the initial synaptic dysfunction theorised to precede synaptic loss in synucleinopathies.

9. Fusion Inhibitors: Tomosyn-2 and PRRT2

9.1 Tomosyn-2

Tomosyn-2 (or syntaxin binding protein 5-like, STXBP5L), a paralog of tomosyn-1 (also known as STXBP5) (Groffen *et al.* 2005) is a large multidomain protein with 14 WD40 repeats and a C-terminal SNARE motif (Williams *et al.* 2011) that is key to its inhibitory actions (Fig. 1). Tomosyn-2 is expressed at high levels in the hippocampus, thalamus and cerebellum, but absent from the cerebral cortex, basal ganglia and amygdala (Groffen et al. 2005). In human brain and spinal cord tissue tomosyn-2 is expressed at a higher level than tomosyn-1 (Kumar *et al.* 2015).

Relatively little is known regarding the molecular mechanisms of tomosyn-2, but information can be gleaned from the closely related tomosyn-1. Tomosyn competes with Munc18-1 for binding to syntaxin-1 in *in vitro* assays (Fujita *et al.* 1998) and the C-terminal VAMP-like SNARE motif of tomosyn forms a tight complex with the t-SNAREs that blocks entry of synaptobrevin-2 into the SNARE complex (Pobbati *et al.* 2004, Li *et al.* 2018). This inhibitory action of tomosyn-1 on SNARE complex assembly is overcome by NSF/αSNAP (Li et al. 2018). However, the SNARE motif is not sufficient for the inhibitory action of tomosyn – the WD40 domain is also required (Yizhar *et al.* 2007). This domain has been shown to catalyse the oligomerisation of the tomosyn SNARE motif and also binds syaptotagmin-1 (Sakisaka *et al.* 2008, Yamamoto *et al.* 2010).

Tomosyn-1 is a negative regulator of initial release probability. Knockdown of tomosyn-1 leads to a larger readily-releasable and recycling pool (Cazares *et al.* 2016). Tomosyn-1 mutant *C. elegans* have increased evoked and sucrose-mediated release (McEwen *et al.* 2006), and an increase in both docked synaptic vesicles and those tethered close to the plasma membrane (Gracheva *et al.* 2010, Gracheva

et al. 2006), demonstrating that tomosyn-1 is a negative regulator of docking/priming. It is thought to do this in a manner antagonistic to Munc13-1 and Munc18 (Gracheva et al. 2010, McEwen et al. 2006). Overexpression of tomosyn-2 in PC12 cells inhibits evoked release to a similar extent as tomosyn-1 (Williams et al. 2011). There is an increase in spontaneous release and faster short term depression at the neuromuscular junctions of tomosyn-2 KO mice; however, hippocampal cultures from these mice displayed no defects in neurotransmission (Geerts *et al.* 2015).

9.1.1 STXBP5L-associated infantile-onset neurodegenerative disorder

A single homozygous mutation in tomosyn-2 (V1043I) has been identified in siblings with a fatal infantile-onset neurodegenerative disorder, presenting with sensorimotor axonal neuropathy and marked cognitive delay prior to progressive cognitive decline (Kumar et al. 2015). It should be noted that compound heterozygous missense variants in another gene also segregated with the disease, but the strong weight of evidence suggests against this being the cause of the disorder. The V1043I mutation is located within the final WD40 repeat of tomosyn-2. This variant inhibited calcium-dependent exocytosis when introduced into PC12 cells, and was a more potent inhibitor of KCl-stimulated release than WT tomosyn-2 (Kumar et al. 2015), suggesting this is a gain-of-function variant.

9.2 PRRT2 paroxysmal disorders

Very little was known regarding proline-rich transmembrane protein 2 (PRRT2) until it was discovered to be mutated in a variety of paroxysmal disorders (which have a sudden onset or sudden exacerbation of symptoms, such as seizures and migraine). PRRT2 mutations are the leading cause of the most common type of familial paroxysmal movement disorder, paroxysmal kinesigenic dyskinesia (PKD, OMIM 128200), as well as benign familial infantile seizures (BFIS, OMIM 605751) and PKD with infantile convulsions (PKD/IC; also known as infantile convulsions with choreoathetosis, ICCA, OMIM 602066). Mutations in PRRT2 also cause other paroxysmal disorders, such as childhood epilepsies, paroxysmal hypnogenic dyskinesia, paroxysmal torticollis, migraine, hemiplegic migraine and episodic ataxia (for review, see (Valtorta *et al.* 2016, Ebrahimi-Fakhari *et al.* 2018)).

Over 90 autosomal dominant mutations (with incomplete penetrance) in PRRT2 have been identified across the whole length of the protein. However, there is a surprising pleiotropy and lack of genotype-phenotype correlation: a single mutation can cause multiple distinct paroxysmal disorders, even within a single family. <1% of cases harbour homozygous or compound heterozygous PRRT2

mutations, which tend to present with a mixed and/or more severe phenotype that can include developmental delay (Ebrahimi-Fakhari et al. 2018) .

Discovery of the key role that PRRT2 plays in these unusual disorders sparked intense interest into its physiological function. PRRT2 is highly enriched at the presynaptic plasma membrane and is also present on small synaptic vesicles (Valente *et al.* 2016, Tan *et al.* 2018). Expression of PRRT2 increases postnatally in parallel with synaptogenesis, and in rodents it is most highly expressed in the lower hindbrain, cerebellum, neocortex, and spinal cord, particularly in areas involved in processing sensory information (Valente et al. 2016, Michetti *et al.* 2017). It is expressed in both excitatory and inhibitory neurons, but is more widely expressed in excitatory synapses (Valente *et al.* 2019). PRRT2 consists of a proline-rich domain within the unstructured N-terminal cytoplasmic region, two transmembrane domains linked by a small cytosolic loop and a short extracellular C-terminal tail (Rossi *et al.* 2016) (Fig. 1). PRRT2 binds SNARE proteins (predominantly syntaxin-1A and SNAP-25) and synaptotagmin-1/-2 (Valente et al. 2016, Tan et al. 2018).

PRRT2 blocks trans-SNARE complex assembly *in vitro* and inhibits the docking and synaptotagmin-dependent fusion of proteoliposomes (Coleman *et al.* 2018), suggesting that PRRT2 is a negative regulator of vesicle priming and fusion. In support of this, there is an increase in docked synaptic vesicles in PRRT2-deficient synapses (Valente et al. 2019, Va*lente e*t al. 2016, Mo et al. 2019). However, the role of PRRT2 in the control of neurotransmitter release is less clear, with PRRT2 depleted cultures and rodents displaying opposing effects on spontaneous and evoked excitatory and inhibitory release (Valente et al. 2019, Valente et al. 2016, Mo et al. 2019). PRRT2 KO rats have a higher excitatory/inhibitory ratio in the M1 motor cortex (Mo et al. 2019), and acute slices from PRRT2 KO mice showed a higher rate of spontaneous network activity and burst firing (Valente et al. 2019). Therefore, PRRT2 may have cell- and region- specific effects, and, given that mutations in PRRT2 result in a variety of paroxysmal disorders, it is perhaps unsurprising that it likely regulates network activity and excitatory/inhibitory balance.

Most mutations in PRRT2 are loss-of-function variants, either truncating mutations subject to nonsense-mediated decay (Valtorta et al. 2016) or interfering with the correct subcellular targeting of the protein (Ma *et al.* 2018, Tsai *et al.* 2019). As such, clinical insight can be drawn from animal models of PRRT2 haploinsufficiency. PRRT2 KO pups display paroxysms (such as bouncing and back walking) while adult mice have loss of balance and exhibit audiogenic seizures characterised by explosive, uncontrolled movements without accompanying alterations to EEG (Michetti et al. 2017). PRRT2 KO rats also display mild motor impairments and increased susceptibility to pentylenetetrazole (PTZ)-induced seizures (Mo et al. 2019). PRRT2 heterozygous rodents, which should more closely model the

heterozygous nature of most PRRT2 mutations, have been less thoroughly characterised, and display very mild bouncing phenotypes (Michetti et al. 2017). Further research is required on heterozygous animal models to ascertain whether they can model PRRT2-related paroxysmal disorders.

In addition to the knockout models, specific mutations in PRRT2 have also been investigated. Homozygous knock-in mice carrying the most common human PRRT2 variant, c.649dupC (a frame-shift mutation subject to nonsense-mediated decay), recapitulates the phenotype of PRRT2 KO mice (Pan *et al.* 2020), though this only models rare, severe cases of homozygous PRRT2 paroxysmal disorders. The PKD mutant G305W, which presents with a comparably more severe clinical phenotype, cannot inhibit SNARE complex assembly and recapitulates the effect of absence of PRRT2, demonstrating that this is also a loss-of-function mutation (Coleman et al. 2018).

In summary, mutations in PRRT2 are likely to result in haploinsufficiency or loss of function of the protein, resulting in an increase in docked vesicles due to increased SNARE complex assembly, leading to cell- and region-specific alterations to neurotransmission and network activity. However, much is left to be discovered regarding the precise role of this protein, and how mutations in PRRT2 can produce such pleiotropic outcomes. It should also be noted that PRRT2 may have other functions which contributes to these disorders (Fruscione *et al.* 2018). Importantly, it is only through discovering that mutations in PRRT2 cause paroxysmal disorders that this protein's role in regulating fusion has been appreciated.

10. Complexin-1

The multifaceted (and contentious) roles of complexins in the regulation of SNARE complexes and neurotransmission have been intensely investigated. Complexin-1 is the major brain isoform, with its expression broadly overlapping with complexin-2 (though there is little complexin-1 in the striatum or outer layers of the cerebral cortex) (Reim *et al.* 2001). Complexins-3 and -4 are predominantly expressed at retinal ribbon synapses. Complexin-1 has an N-terminal domain, an accessory α-helix, a central α-helix, and a C-terminal domain (Fig. 1). The central α-helix of complexin forms part of the tripartite interface of the "primed, locked" synaptotagmin-complexin-SNARE complex (Zhou et al. 2017). The C-terminal domain of complexin, which is poorly understood in terms of complexin function, contains a putative amphipathic helical domain that mediates localisation of the protein and synaptic vesicle binding (Gong *et al.* 2016), and helps stimulate fusion of highly curved membranes (Malsam *et al.* 2009).

Complexin has at least two functions in regulating the fusion of synaptic vesicles; however, these functions are in part species- and/or cell-type specific. Debate has long raged regarding the ability of complexin to clamp spontaneous release (for example, see reviews (Rizo 2018, Trimbuch & Rosenmund 2016)), and differences likely arise from the model system used. Additionally, mammalian and invertebrate complexins may act differentially (Wragg *et al.* 2017), with complexin potently clamping spontaneous release in invertebrates (Trimbuch & Rosenmund 2016), while the evidence is less clear-cut in mammals. Spontaneous release was increased in cultured complexin-1/2 knockdown cortical neurons (Maximov *et al.* 2009), but was unaffected in cultures from complexin-1, -2 and double complexin-1/2 KO mice (Reim et al. 2001). Subsequently, it was shown that complexin-1/2 and -1/2/3 KO cultures and acute slices display reduced spontaneous excitatory (but not inhibitory) release (Xue *et al.* 2008b), refuting a role for mammalian complexin-1 as a clamp of spontaneous neurotransmitter release. Instead, synaptotagmin-1 is likely to be the fusion clamp in mammalian neurons (Courtney *et al.* 2019).

The evidence for complexin-1 as an activator of Ca^{2+}-dependent synchronous release is much stronger. Evoked release is greatly reduced in cultured hippocampal neurons from complexin-1/2 KO mice but was not affected by knockout of either complexin-1 or -2 in isolation (Reim et al. 2001). In complexin-1/2/3 null hippocampal cultures there is a strong decrease in the rate of all forms of synaptic vesicle fusion (spontaneous, evoked synchronous and asynchronous fusion) (Lopez-Murcia *et al.* 2019). This supports the concept that complexin regulates vesicle fusogenicity in mammalian cells by lowering the energy barrier for primed vesicles to undergo either Ca^{2+}-evoked or spontaneous fusion (Trimbuch & Rosenmund 2016). Recent studies suggest that complexin-1 does not affect priming (Lopez-Murcia et al. 2019) but limits de-priming by protecting the assembled trans-SNARE complex from disassembly (Prinslow et al. 2019) through competition with αSNAP for SNARE complex binding (Choi *et al.* 2018).

10.1 CPLX1-early infantile epileptic encephalopathy

A limited number of individuals carrying homozygous mutations in the gene encoding complexin-1 (*CPLX1*) have been identified presenting with early infantile epileptic encephalopathy (OMIM 617976). They include two siblings with malignant migrating epilepsy and cortical atrophy, harbouring a truncating nonsense mutation (E108*) (Karaca *et al.* 2015) and two siblings with severe epilepsy (likely cause of death in one case), global developmental delay, and intellectual disability, with a nonsense mutation in a nearby residue (C105*) (Redler *et al.* 2017). Finally, an individual with a missense mutation (L128M) in the C-terminal domain of complexin-1 presented with persistent generalised

seizure activity and developmental delay (Redler et al. 2017). These homozygous mutations all lie within the final exon, affecting the C-terminal domain of the protein.

None of these mutations have been specifically examined in *in vitro* or *in vivo* assays. L128 is thought to play a key role in membrane binding of complexin-1 (Wragg et al. 2017), and alteration of this residue may thus impede correct targeting of the protein. The other variants are predicted to cause truncation of the C-terminal domain of complexin-1, and may also result in loss of function due to mislocalisation, as deletion of the C-terminal domain diminishes the ability of complexin to be targeted to synapses (Gong et al. 2016). Therefore, mammalian systems lacking complexin-1 may provide insights into pathogenesis of this disorder.

Evoked synchronous, spontaneous, and sucrose-mediated release are all normal in complexin-1 KO autaptic hippocampal cultures, as are the number of docked vesicles and vesicle density (Reim et al. 2001). Despite loss of complexin-1 having no overt effects on neurotransmission (likely resultant from compensation by complexin-2), comparatively more severe effects are seen at the systems level in rodent models lacking complexin-1. Complexin-1 KO mice and rats die 2-4 months after birth, are profoundly ataxic, exhibit dystonia and resting tremor, have motor impairments, and, in line with epilepsy observed in patients, some mice have sporadic seizures (Reim et al. 2001, Xu et *al. 20*20, Glynn *et al.* 2005). However, contrary to the clinical phenotype, complexin-1 KO rodents have no evidence of atrophy or severe cognitive deficits, with only mild changes in sociability, impulsivity, exploratory behaviour, and agitation (Reim et al. 2001, Xu et al. 2020, Glynn e*t al. 2*005, Drew et al. 2007). This suggests that there may be species differences in the importance of complexin-1 for cognition.

11. Ca^{2+} sensors: Synaptotagmins

Synaptotagmins are a family of double C2 domain-containing transmembrane proteins, 17 of which are found in the human genome (Craxton 2007). Synaptotagmin-1, -2 and -9 drive calcium-dependent fast synchronous release (Geppert *et al.* 1994, Fernandez-Chacon *et al.* 2001, Pang *et al.* 2006a, Xu *et al.* 2007), while synaptotagmin-7 mediates asynchronous release (Wen *et al.* 2010, Volyn*ski & K*rishnakumar 2018, Bacaj et al. 2013). The role of the other family members is much less well known and, of the synaptic vesicle synaptotagmins, disease-causative mutations have only been found in synaptotagmin-1 and -2.

11.1 Synaptotagmin-1

Synaptotagmin-1 (first identified as p65), the best characterised family member, is the major Ca^{2+} sensor for evoked synchronous release in the brain. It is most highly expressed in the cerebral cortex, hippocampus, striatum, midbrain, and sensory and autonomic peripheral nerves (Ul*rich e*t al. 1994, Ul*rich &* Sudhof 1995, L*i et al*. 1994, Banerjee et al. 2020, Marqueze *et al.* 1995). Synaptotagmin-1 consists of a short intravesicular N-terminal domain, single transmembrane region, charged linker region, and two tandem C2 domains (C2A and C2B) (Perin *et al.* 1991a, Perin *et al.* 1991b) (Fig. 1).

Two synatotagmin-1 molecules bind to the complexin-SNARE complex at two distinct interfaces, the primary interface and the tripartite interface (Zhou et al. 2017, Zhou et al. 2015), and this multi-protein complex likely comprises the primed and "locked" state of vesicles. Synaptotagmin-1 also helps protect against NSF-αSNAP-mediated disassembly of trans-SNARE complexes (Prinslow et al. 2019). The C2B domain of synaptotagmin-1 is essential for clamping of spontaneous release (Courtney et al. 2019), which may involve oligomerisation of the C2B domains (Tagliatti *et al.* 2020); this may also be important for clamping asynchronous release (Tagliatti et al. 2020). There are five key aspartate residues in both C2 domains that coordinate three (C2A) or two (C2B) Ca^{2+} ions. Ca^{2+}-binding neutralises the charge of the C2 domains to facilitate membrane penetration by synaptotagmin-1 (Paddock *et al.* 2011, Bowers & Reist 2020), which relieves clamping of release and allows the trans-SNARE complex to fully zipper, thereby triggering fusion (for review of this model see Brunger et al. (2019)). Membrane penetration by synaptotagmin-1 is also likely to directly facilitate fusion (Bowers & *Reist 2*020, Kiessling et al. 2018).

Synaptotagmin-1 is essential for life, as KO mice die within 48 hours of birth. Cultured hippocampal or cortical neurons from synaptotagmin-1 KO mice have deficits in action potential-evoked and sucrose-mediated release at excitatory and inhibitory synapses, in addition to potentiation of spontaneous release (Courtney et al. 2019, Geppert et al. 1994, Maxim*ov & Su*dhof 2005, Liu *et al.* 2009, Chi*cka et* al. 2008, Pang et al. 2006b). Synaptotagmin-1 KO cultures also have a decrease in morphologically docked vesicles and release probability (Liu et al. 2009, Imig et al. 2014).

11.1.1 Baker-Gordon Syndrome

Heterozygous mutations in *SYT1*, encoding synaptotagmin-1, cause a neurodevelopmental disorder known as Baker-Gordon Syndrome (or *SYT1*-associated neurodevelopmental disorder, OMIM 618218). This syndrome consists of a phenotypic spectrum of moderate to severe intellectual disability, infantile hypotonia, motor delay, ophthalmic abnormalities and episodic agitation. All affected individuals have disturbed EEG recordings but no record of epileptic seizures, and some cases

developed childhood-onset hyperkinetic movement disorders (Baker *et al.* 2015, Baker *et al.* 2018). 5 distinct *de novo* variants have been identified in 11 patients, with cases of recurrent mutations presenting similarly. Two cases were reported in Bradberry *et al.* (2020) but refer to the same individuals identified in Baker et al. (2018). All variants are missense mutations (M303K, D304G, D366E, N371K and I368T) in conserved residues that cluster around the Ca^{2+}-binding pocket of the C2B domain (Baker et al. 2018), which would be expected to perturb Ca^{2+}-dependent synaptic vesicle fusion.

D304 and D366 are two of the five key aspartates in the C2B domain that directly bind Ca^{2+}, and I368 is the hydrophobic tip residue required for membrane penetration (Paddock et al. 2011), which would be impeded by substitution of a hydrophilic threonine (I368T). All variants were found to express and localise to presynaptic terminals similarly to the WT protein in cultured mouse hippocampal neurons, except for M303K (Baker et al. 2018). Molecular dynamics simulations suggested that M303K substitution could alter the structure of the C2B domain and thereby possibly impact expression, stability or trafficking of the protein (Baker et al. 2018), whereas overall structure is unlikely affected by D304G, D366E, I368T and N371K mutations (Baker et al. 2018, Bradberry et al. 2020). However, simulations showed that D304G would significantly impair Ca^{2+} binding (Baker et al. 2018). D304G, D366E and I368T were demonstrated to reduce Ca^{2+}-dependent membrane binding by synaptotagmin-1 *in vitro*, with D304G producing the most severe effect (Bradberry et al. 2020).

Overexpression of synaptotagmin-1 mutants in WT hippocampal cultures, modelling the heterozygous condition, slowed synaptic vesicle exocytosis, indicating a dominant-negative mechanism of disease (Baker et al. 2018, Baker et al. 2015). D366E inhibited the rate of fusion to a lesser degree than the D304G, I368T and N371K variants (Baker et al. 2018). This effect was recapitulated (for D304G, D366E, and I368T variants) at inhibitory and excitatory synapses in a different heterozygous system (Bradberry et al. 2020). Oligomerisation is important for synaptotagmin-1 function (Tagli*atti et* al. 2020, Bello et al. 2018), and may mediate these dominant-negative effects.

To investigate whether these mutations affect the functionality of synaptotagmin-1, variants D304G, D366E and I368T were expressed in cultured cortical neurons from synaptotagmin-1 KO mice, where all failed to rescue action potential-evoked inhibitory release (Bradberry et al. 2020). The kinetics of the residual release were also slowed to the same extent as synaptotagmin-1 KO^{-} for D304G and I368T, whereas D366E maintained faster release kinetics closer to those seen in the WT rescue. D304G also failed to clamp spontaneous release, resembling the increase in spontaneous fusion seen in synaptotagmin-1 KO neurons, while D366E and I368T retained some clamping ability, albeit less than the WT protein (Bradberry et al. 2020). However, clamping of spontaneous release was not

investigated in a heterozygous model so it is unknown if this contributes to synaptic dysfunction in this disorder.

With the exception of M303K, all synaptotagmin-1 mutations have been demonstrated to compromise evoked synaptic vesicle fusion, which likely constitutes the primary pathogenic mechanism of these variants. Interestingly, the recurrent D366E mutation is associated with a milder phenotype (Baker et al. 2018) and was consistently found to produce a less severe impairment of exocytosis (Baker et al. 2018, Bradberry et al. 2020). This suggests a genotype-phenotype relationship where variation in patient phenotype may reflect mutation-specific impact on synaptic vesicle fusion.

11.2 Synaptotagmin-2

Synaptotagmin-2 (encoded by *SYT2*) is another major Ca^{2+} sensor mediating fast synchronous neurotransmitter release that is highly homologous to synaptotagmin-1 (Geppert *et al.* 1991, Pang et al. 2006a). Synaptotagmin-2 is predominantly expressed in caudal regions of the central nervous system (including the cerebellum, brainstem and motor neurons of the spinal cord), but is also expressed in discrete populations of (particularly inhibitory) forebrain neurons (Chen et al. 2017, Mittelsteadt et al. 2009, Ullrich et al. 1994, Ullrich *& Sudho*f 1995, Bragina et al. 2011, Marqueze *et al.* 1995, Sommeij*er & Le*velt 2012, Pang et al. 2006a, Berton *et al.* 1997). A developmental switch from synaptotagmin-1 to -2 has been observed in motor neurons and the calyx of Held (Berton et al. 1997, Kochubey *et al.* 2016, Campagna *et al.* 1997). Accordingly, synaptotagmin-2 KO mice are viable at birth but in the second postnatal week they develop motor impairments and exocytic defects at the neuromuscular junction, followed by death around three weeks of age (Pang et al. 2006a). Synaptotagmin-2 therefore plays an essential role for in neuromuscular transmission and survival.

11.2.1 Synaptotagmin-2 Congenital Myasthenic Syndrome

Both dominant and recessive mutations in synaptotagmin-2 (encoded by *SYT2*) have been found to cause a presynaptic congenital myasthenic syndrome (CMS7, OMIM 616040) of differential severity. To date, clinical reports have been published on 20 individuals with 9 distinct mutations (Herrmann *et al.* 2014, Montes-Chinea *et al.* 2018, Maselli *et al.* 2020, Donkervoort *et al.* 2020). Heterozygous missense mutations manifest as a hereditary distal motor neuropathy with common symptoms including childhood-onset foot deformities, distal limb weakness, areflexia and gait abnormalities (Herrmann et al. 2014, Montes-Chinea et al. 2018). An electromyographic hallmark of this condition is low amplitude compound muscle action potentials with post-exercise facilitation (Whittaker *et al.*

2015). In contrast, for individuals harbouring homozygous mutations, symptoms present at or before birth and include: severe hypotonia, severe motor delay and profound axial, proximal and facial muscle weakness with respiratory involvement in some cases (Donkervoort et al. 2020, Maselli et al. 2020).

Similarly to synaptotagmin-1, all heterozygous missense mutations (D307A, P308L and I371K) in synaptotagmin-2 are situated around the Ca^{2+}-binding pocket of the C2B domain (Herrmann et al. 2014, Montes-Chinea et al. 2018). While D307A substitutes one of the well-researched Ca^{2+}-binding aspartates in the C2B domain, the significance of the other substituted residues is unknown; hence, these disease-associated variants represent the first evidence for the importance of P308 and I371 in synaptotagmin. Molecular modelling predicted that the P308L mutation would distort the Ca^{2+}-binding loop in which it lies (Shields *et al.* 2017).

Separate functional studies have been performed on the three heterozygous missense variants, where the corresponding mutations were induced in drosophila synaptotagmin-1 and examined at larval neuromuscular junctions (Shields et al. 2017, Herrmann et al. 2014, Montes-Chinea et al. 2018). Cursory inspection showed that mutant proteins all express similarly to the WT protein and localise to neuromuscular junctions. In synaptotagmin-1 KO larvae, expression of D307A or I371K mutants failed to rescue evoked synchronous release, or clamp asynchronous or spontaneous release, unlike the WT protein (Montes-Chinea et al. 2018, Herrmann et al. 2014).

However, the human disorder is better modelled by a heterozygous system. In fact, drosophila heterozygous for the P308L variant exhibited impaired motor activity and fast rate of fatigue, somewhat mirroring patient symptoms (Shields et al. 2017). When expressed alongside endogenous synaptotagmin, D307A, P308L and I371K mutants reduced evoked neurotransmitter release in a dominant-negative manner, though P308L to a lesser degree. All three mutants were also demonstrated to lower the probability of vesicular release (Herrmann et al. 2014, Montes-Chinea et al. 2018, Shields et al. 2017). The frequency of spontaneous fusion events was also increased for D307A, indicating failure of synaptotagmin-1-mediated clamping (Herrmann et al. 2014); however, for P308L neither spontaneous nor sucrose-evoked release were altered (Shields et al. 2017).

In contrast, the very recently identified homozygous synaptotagmin-2 variants include nonsense, frameshift and deletion mutations (Exon 3-9 deletion, V243Gfs*13, E269*, Y309*, R397Sfs*37)(Donkervoort et al. 2020, Maselli et al. 2020). These loss-of-function mutations are not deleterious in the heterozygous state, as carrier parents are unaffected. No functional assays have investigated the specific effect of patient biallelic loss-of-function mutations in synaptotagmin-2, but insight can be gathered from a KO mouse model. A reduction in synchronous action potential-evoked

release, desynchronization of release and substantial increase in the frequency of spontaneous release is found at the neuromuscular junction of synaptotagmin-2 KO mice (Pang et al. 2006a).

One homozygous frameshift mutation (R397Sfs*37) may potentially escape nonsense-mediated decay as it replaces the C-terminal 23 residues of synaptotagmin-2 with an erroneous 37 amino acid sequence that supplants three key features of the C2B domain structure: one of two crucial exposed arginines (R396 and R397) on the "bottom" calcium-independent face, β-strand 8, and the C-terminal α-helix (also known as HB) (Maselli et al. 2020). These regions form part of the primary- and tripartite interfaces in the synaptotagmin-complexin-SNARE complex, and R397 is additionally crucial for synchronous neurotransmitter release (Zho*u et al*. 2015, Zhou et al. 2017, Xue et al. 2008a).

Alternatively, it is important to note that truncation, internal deletion and mutation of highly conserved C-terminal amino acids of synaptotagmin-1 or -2 has caused mislocalisation and compromised internalisation of the protein in PC12 cells (Blagoveshchenskaya *et al.* 1999, Fukuda *et al.* 2000, Krasnov & Enikolopov 2000, Jarousse *et al.* 2003), though this has not been assayed in neurons. This suggests that the C-terminal tail of synaptotagmin-2 contains important trafficking and/or endocytic motifs and that this frameshift mutation could cause mislocalisation, and therefore deficiency, of synaptotagmin-2. Furthermore, the sizeable disruption induced by this frameshift mutation, encompassing an entire β-strand, could destabilise the C2B domain resulting in loss of synaptotagmin-2 function and/or expression; this model would be in line with the recessive nature of the disorder caused by this variant and is supported by the similar presentation of other newly identified homozygous loss-of-function mutations.

12. Toxins that alter Ca^{2+} influx

It is now evident that synaptic vesicle fusion is under tight control, and even small alterations to the machinery that regulates this can lead to neurological dysfunction. Hence, it is unsurprising that nature has developed a variety of ways to circumvent this tight control as means of both defence and attack. Thus, toxins that alter Ca^{2+} influx can be considered to cause acute disorders of fusion, and research using these toxins has contributed greatly to our fundamental understanding of synaptic vesicle fusion.

12.1 Toxins that act upon voltage-gated Ca^{2+} channels

Many toxins alter Ca^{2+} influx through direct action on voltage-gated Ca^{2+} channels, including those channels primarily responsible for generating local Ca^{2+} nanodomains at the presynaptic active zone: $Ca_v2.1$ (P/Q-type channels), $Ca_v2.2$ (N-type channels) and $Ca_v2.3$ (R-type, which plays a more limited and variable role) (Ricoy & Frerking 2014). These toxins will not be discussed in depth here, and we note only a few examples acting on Ca^{2+} channels via distinct mechanisms and are components of venoms of medical significance to humans (for review see Bourinet and Zamponi (2017)).

Isolated from the venom of *Conidae* marine snails, which are a rare cause of human injury and death (Kohn 2016), ω-conotoxins primarily target N-type ($Ca_v2.2$) or P/Q-type (CaV2.1) Ca^{2+} channels, where they act to physically occlude the channel pore and thereby block Ca^{2+} influx (Ovsepian et al. 2019, Bourinet & Zamponi 2017). These have been increasingly gaining interest as putative therapeutic agents (Lewis 2009, Hannon & Atchison 2013). The venom of the Brazilian wandering spider or armed spider (*Phoneutria nigriventer)* contains a cocktail of toxins which act on a variety of ion channels (Gomez *et al.* 2002). A bite from this highly aggressive spider can cause serious illness (including cramps, tonic convulsions and spastic paralysis) (Gomez et al. 2002). One component from this venom, PhTx3-3, abolishes Ca^{2+}-dependent neurotransmitter release (Prado *et al.* 1996). It blocks Ca^{2+} influx by broadly inhibiting high voltage Ca^{2+} channels, with a preference for P/Q and R-channels (Leao *et al.* 2000). PhTx3-4 and 3-6 also block evoked Ca^{2+} influx into nerve terminals and similarly target N-, P/Q-, and R-type Ca^{2+} channels (Vieira *et al.* 2005, Dos Santos *et al.* 2002). Finally, kurtoxin, from the venomous scorpion *Parabuthus transvaalicus*, acts as a high affinity gating modifier across several different Ca^{2+} channel types, including N- and P-type channels (Sidach & Mintz 2002). The extremely painful sting from *Parabuthus transvaalicus* is relatively common in parts of Africa and can cause neuromuscular impairments and may result in death (albeit rarely) (Bergman 1997).

12.2 Toxins that circumvent regulation of Ca^{2+}-dependent fusion

12.2.1 Latrotoxin

α-latrotoxin, a component of venom from the widow family of spiders (black and brown widow, red-back spiders), causes an increase in spontaneous neurotransmitter release and a concomitant depletion of synaptic vesicles from terminals (Longenecker et al. 1970, Clark et al. 1970, Cull-Candy *et al.* 1973). In fact, it was this connection that synaptic vesicles were lost as neurotransmitter release occurred that gave credence to synaptic vesicles being the biophysical correlates of quanta for neurotransmission. Poisoning with α-latrotoxin can cause latrodectism, with symptoms including

muscle pain, abdominal cramps, sweating, increased blood pressure and tachycardia, and can result in death, albeit rarely (Ovsepian et al. 2019).

Incredibly, even after decades of intensive research, the molecular mechanisms underlying α-latrotoxin's toxicity remain debated. Following binding to its cell surface receptors neurexin and/or latrophilin (also called CIRL, Ca^{2+}-independent latrotoxin receptor) (Silva *et al.* 2009), α-latrotoxin has been suggested to induce its toxicity via two distinct mechanisms. Firstly, latrotoxin can directly induce toxicity by forming a stable homotetrameric cation-conductive channel, creating a pore in the membrane with high inward conductance for Ca^{2+} and Mg^{2+}, thereby triggering synaptic vesicle fusion (Silva et al. 2009, Finkelstein et al. 1976, Wanke *et al.* 1986, Robello *et al.* 1987, Volynski *et al.* 2000, Orlova *et al.* 2000). It has been suggested that the fusion pore might function in a similar manner to hypertonic sucrose (Silva et al. 2009), but unlike sucrose-mediated fusion, this can occur even in the absence of synaptobrevin-2, SNAP-25, Munc13-1 and synaptotagmin-1 (Pumplin & del Castillo 1975, Geppert et al. 1994, Deak *et al.* 2009), and may also involve non-vesicular diffusion of cytoplasmic neurotransmitters directly through the pore (Davletov *et al.* 1998). As a potential second mechanism of action, α-latrotoxin can induce neurotransmitter release by activating receptor-mediated signalling cascades. This is likely dependent on latrophilin, an adhesion GPCR, which is normally cleaved into two fragments. α-Latrotoxin acts as an exogenous ligand causing re-association of the protein and subsequent intracellular Gαq-mediated signalling (Ushkaryov *et al.* 2019), resulting in mobilisation of intracellular Ca^{2+} stores (Capogna *et al.* 2003, Davletov et al. 1998). However, the importance of this is contentious, as deletion of the cytoplasmic region of latrophilin responsible for G-protein coupling, had no impact on α-latrotoxin-induced hormone release in PC12 cells (Sugita *et al.* 1998). The receptor-mediated induction of neurotransmitter release by latrotoxin is dependent on SNARE proteins (Deak et al. 2009) and synaptotagmin-1 (Shoji-Kasai *et al.* 1994).

12.2.2 Trachynilysin and other related venoms

The highly toxic stonefish (*Synanceia trachynis*) venom (trachynilysin), which can be fatal to humans, was shown to cause an increase in neurotransmitter release at the frog neuromuscular junction, and depletion of synaptic vesicles (Kreger *et al.* 1993). Trachynilysin causes an increase in intracellular Ca^{2+} (Meunier *et al.* 2000) most likely by forming pores in membranes (Ouanounou *et al.* 2002). The crude venoms of the soldierfish (*Gymnapistes marmoratus*) and the lionfish (*Pterois volitans*), which also display toxicity to humans, albeit less so than stonefish venom, display pronounced neuromuscular activity, and are likely to work via similar mechanisms to trachynilysin (Church *et al.* 2003). The ability

of La^{3+} to block the activity of these toxins suggest that they form Ca^{2+}-permeable pores in a similar manner to latrotoxin.

13. Summary and Remaining Questions

In order to safeguard neurotransmitter release, there is a great deal of redundancy in fusion proteins, where loss of one family member does not completely abolish synaptic vesicle exocytosis. However, neurological disorders associated with mutations in these proteins highlight that effective neurotransmission in the human brain requires extremely efficient fusion mechanisms and, despite this redundancy, minor perturbations can have serious consequences on neurological function.

Investigations into the pathogenicity of neurological disorders has driven our understanding of the molecular mechanisms underlying synaptic vesicle fusion. For example, it is only through identifying PRRT2 and α-synuclein as genetic causes of movement disorders that interest in their presynaptic functions was sparked, and indeed, it was the discovery of the targets and mechanisms of action of neurotoxins that informed our understanding of synaptic vesicle fusion. Now, during the genetic revolution, we are afforded even deeper insight into these processes, at the domain and even single residue level. For example, a disease-associated mutation in Munc13-1 lies in a region of the protein of unknown significance and, intriguingly, increases fusion. Such a finding is certainly unexpected, and only made possible by dissecting protein function through the lens of human disorders.

Currently, the capacity to categorise and compare these disorders is limited by inconsistencies in the level of detail of patient symptoms, terminology and classification criteria used in the reporting papers, calling for comprehensive and standardised clinical characterisation. However, with this caveat in mind, some themes emerge from our investigation (Fig. 2):

1) Central SNARE proteins (synaptobrevin-2, syntaxin-1B and SNAP-25), Munc18-1 and complexin-1 cause a common phenotypic spectrum of developmental delay, intellectual disability, epilepsy and often movement disorders. Munc13-1 and tomosyn-2 may also align with this group, however, further cases will be required in order to determine common phenotypic features.
2) Although they share symptoms, mutations in SNARE proteins do not appear to all cause the same disorder. While synaptobrevin-2 and SNAP-25 disorders significantly overlap, neurological dysfunction caused by mutations to syntaxin-1B is more heterogeneous. It should be noted that there are fewer individuals with synaptobrevin-2 variants, and it is possible that

severe cases have been identified first. Additionally, syntaxin-1B is currently distinct amongst the SNARE proteins in that haploinsufficiency has been demonstrated to account for some variants, notably with milder presentation.

3) We note that seizures are common in disorders of synaptic vesicle fusion. Indeed, only four proteins have not been linked to seizures: α-synuclein, synaptobrevin-1, and synaptotagmin-1 and -2. Moreover, of all fusion proteins causing neurodevelopmental disorders, synaptotagmin-1 is a notable outlier in the absence of any documented seizures (despite abnormal EEG recordings observed in all patients).
4) All fusion proteins are linked to movement disorders (or neuromuscular disorders for peripherally enriched isoforms), including proteins associated with the neurodegenerative disorders and tetanus and botulinum poisoning.
5) Mutations in the SNARE chaperones cause neurodegenerative disorders. Some older individuals with STXBP1-encephalopathies also have Parkinsonian symptoms (Lanoue *et al.* 2019), suggesting that there may be some neurodegeneration at later stages in these disorders, though this requires further clinical evidence. Similarly, information regarding any neurodegeneration associated with Munc13-1 mutations is lacking. However, a homozygous mutation in tomosyn-2 (which may act antagonistically to Munc13 and Munc18) also results in early-onset neurodegeneration. Therefore, defective SNARE chaperoning or assembly may contribute to pathogenesis in neurodegenerative disorders.

Overall, the pattern of protein expression within the brain and nervous system appears to be a major determinant of symptom type. Therefore, comprehensive cataloguing of expression patterns of synaptic vesicle fusion proteins in human tissue (including splice variants) will be important for better understanding the origin of symptoms and roles of distinct brain regions, as well as for predicting reactions to therapeutics. Indeed, there is some evidence that toxins that affect neurotransmitter release could be repurposed to ameliorate genetic disorders of synaptic vesicle fusion (Ovsepian et al. 2019). However, this would only be possible if we first understand the precise expression and mechanisms of the affected proteins.

Future research should also ensure that disease variants are comprehensively examined in neuronal model systems, with emphasis on establishing expression levels and subcellular localisation of variants before functional assays of synaptic physiology are conducted. This basic information is strikingly lacking from many studies, which limits our understanding of the pathogenic mechanisms of the disease. Finally, investigation of the effects of heterozygous variants has often been performed in KO models; while instrumental in informing the function of the affected protein and residues, they

provide less valuable insight into pathogenesis of disease than studies modelling heterozygous expression. We recommend that future investigations are performed hand-in-hand using these two model systems to imbue studies with both translational and reverse-translational value.

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Figure legends

Figure 1: **Presynaptic proteins associated with disorders of synaptic vesicle fusion.** Markers above each protein indicate splice site variants, missense, nonsense, frameshift, and indel mutations associated with disorders of fusion. Dominant mutations are labelled in teal, while recessive mutations

are labelled in purple. Orange markers below proteins indicate the cleavage sites of botulinum neurotoxin (listed by subtype) and tetanus neurotoxin. Protein length is noted at the C-terminal end of each protein. Hash symbols (#) identify proteins for which the abundance of associated mutations have not been depicted. For summary of mutations, see Valtorta et al. (2016) for PRRT2 or Abramov et al. (2020) for Munc18-1. Abbreviations: BoNT, botulinum neurotoxin; CaMb, calmodulin-binding region; CTD, C-terminal domain; H (e.g. Ha), helical domain; NTD, N-terminal domain; SSV, splice site variant; TeNT, tetanus neurotoxin; TM, transmembrane region.

Figure 2: Venn diagram illustrating phenotypic overlap of disorders of synaptic vesicle fusion. Genes are placed according to the features (intellectual disability, seizures, neuromuscular or movement disorder, and/or neurodegeneration) commonly present in their associated disorders. Genes listed multiple times represent distinct disorders associated with that gene. Font colour denotes category of protein function (see key). Asterisks (*) indicate disorders that have only a neuromuscular phenotype, and are not classified as movement disorders. Abbreviations: AD-ANCL, autosomal dominant adult-onset neuronal ceroid lipofuscinosis; BAGOS, Baker-Gordon Syndrome; BFIS, benign familial infantile seizures; CMS, congenital myasthenic syndrome; DEE, developmental and epileptic encephalopathy; EIEE, early infantile epileptic encephalopathy; GEFS+, generalised epilepsy with febrile seizures plus; LBD, Lewy body dementia; MSA, multiple system atrophy; neurodegen., neurodegenerative; neurodev., neurodevelopmental; PD, Parkinson's disease; PKD, paroxysmal kinesigenic dyskinesia; PKD/IC, PKD with infantile convulsions; SPAX, spastic ataxia.

Table 1: Summary of clinical phenotypes of disorders of synaptic vesicle fusion. Cases refers to the number of cases in published literature. Yes = phenotype is strongly associated with the disorder; some = some but not all cases exhibit this phenotype; rare = phenotype is not commonly associated with this disorder but is observed in a small proportion of cases; no **=** phenotype is not associated with the disorder; ? = unknown. Abbreviations: cog. dec., cognitive decline; DMD, developmental motor delay; ID, intellectual disability; neurodegen., neurodegeneration; opth. ab., ophthalmic abnormalities.

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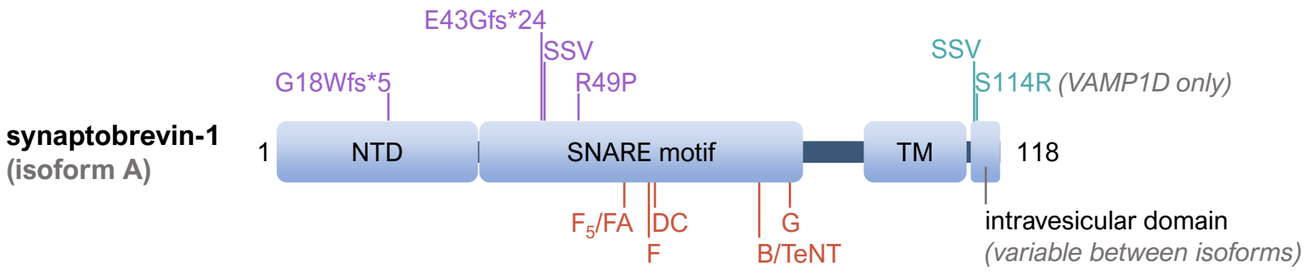
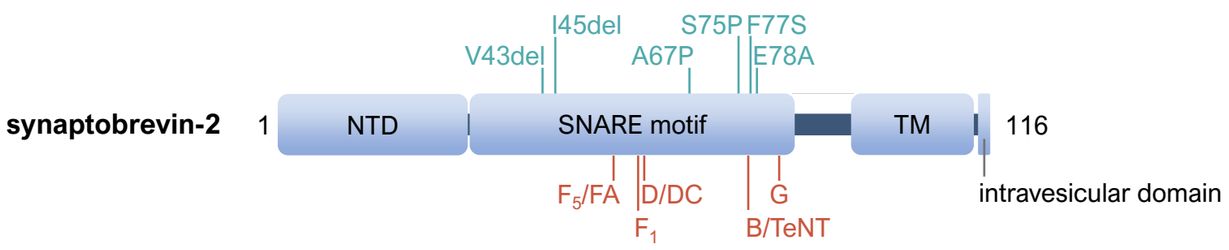
Protein *(GENE)*	Disorder	OMIM#	Cases	Onset	ID	DMD	Seizures	Motor impairments	Muscle weakness	Opth. ab.	Cog. dec.	Neuro-degen.	Other notable features	References
Synaptobrevin-1 ***(VAMP1)***	Autosomal dominant spastic ataxia 1 (SPAX1)	108600	>50	10-20 years	No	No	No	Spasticity, ataxia, gait disturbance, hyperreflexia, hypertonia, dystonia, tremor, head jerks	No	Yes	No	Yes	Degeneration of the corticospinal tracts and posterior columns, loss of midbrain neurons.	(Grewal *et al.* 2004, Bourassa *et al.* 2012)
	Congenital myasthenic syndrome 25 (CMS25)	618323	7	Birth	No	Yes	No	Severe muscle weakness, infantile hypotonia, areflexia, contractures, facial myopathy	Yes	Yes	No	No	One case was fatal.	(Monies *et al.* 2017, Salpietro *et al.* 2017, Shen *et al.* 2017)
Synaptobrevin-2 ***(VAMP2)***	Neurodevelop-mental disorder (unnamed)	618760	6	Birth	Yes	Yes	Some	Axial hypotonia, chorea, dystonia, hyperkinesia	No	Some	No	No	Abnormal behaviour, stereotypies, autistic features, poor visual fixation, absent purposeful hand movements. Intellectual disability is moderate to severe.	(Salpietro *et al.* 2019, Sunaga *et al.* 2020)
SNAP-25 ***(SNAP25)***	Developmental and epileptic encephalopathy (DEE)	616330	22	Infancy to childhood (usually first two years of life)	Yes	Yes	Yes	Areflexia, ataxia, dystonia, hypotonia, motor clumsiness, spasticity, tremor	Rare	Some	No	No	Intellectual disability is mild to profound. Most cases present with seizures.	(Rohena *et al.* 2013, Shen *et al.* 2014, Hamdan *et al.* 2017, Fukuda *et al.* 2018, Klöckner *et al.* 2020)
Syntaxin-1b ***(STX1B)***	Generalized epilepsy with febrile seizures plus 9 (GEFS+ 9)	616172	31	Infancy to childhood	Rare	No	Yes	None	No	No	No	No	Intellectual disability, is absent or mild.	(Wolking *et al.* 2019)
	Developmental and epileptic encephalopathy (DEE)	-	15	Infancy to early childhood	Yes	Yes	Yes	Ataxia	Some	No	Some	No	Intellectual disability is moderate to severe.	(Wolking et al. 2019)

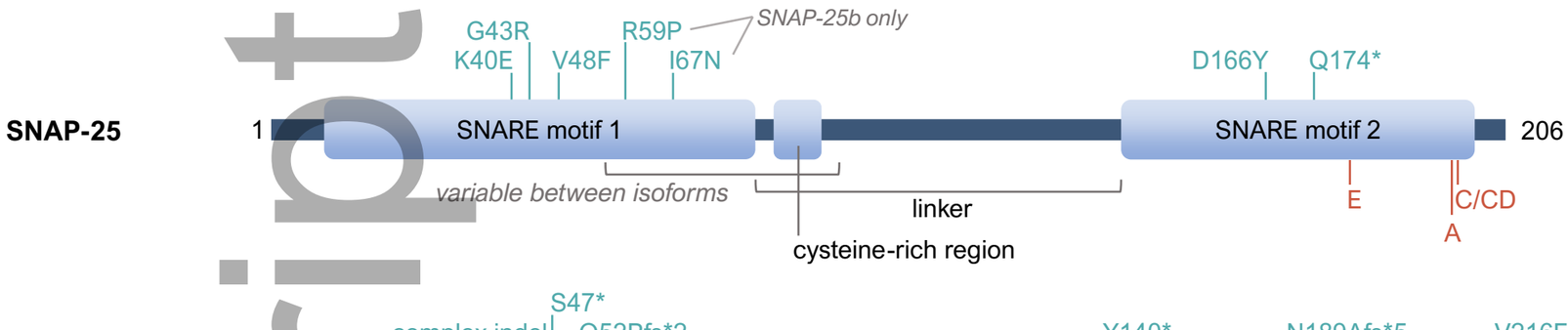
Protein *(GENE)*	Disorder	OMIM#	Cases	Onset	ID	DMD	Seizures	Motor impairments	Muscle weakness	Opth. ab.	Cog. dec.	Neuro-degen.	Other notable features	References
Munc13-1 *(UNC13A)*	Microcephaly, cortical hyperexcitability, fatal myasthenia (unnamed)	-	1	Birth	Yes	Yes	No	Hyporeflexia, hypotonia, minimal movement	Yes	Yes	No	No	Microcephaly, thin corpus callosum, abnormal EEG.	(Engel *et al*. 2016)
	Dyskinetic movement disorder (unnamed)	-	1	First months of life	Yes	Yes	Yes	Dyskinesia (hyperkinesia with continuous movements and intention tremor)	No	No	No	No	Comorbid attention deficit hyperactivity disorder and autism spectrum disorder. 2 febrile seizures (age 4).	(Lipstein *et al.* 2017)
Munc18-1 *(STXBP1)*	*STXBP1* encephalopathy	612164	~200	Infancy (first 12 months)	Yes	Yes	Yes	Variable: ataxia, dyskinesia, hypotonia, spastic di-/quadriplegia, tremor	Some	No	No	?	Highly variable symptoms and severity. Some present with cortical atrophy, thin corpus callosum and/or hypomyelination.	(Deprez *et al*. 2010, Stamberger *et al.* 2016, Abramov *et al*. 2020)
CSPα *(DNAJC5)*	Autosomal dominant adult-onset neuronal ceroid lipofuscinosis (AD-ANCL)	162350	>35	Adulthood	No	No	Yes	Ataxia, myoclonus	No	No	Yes	Yes	Presence of autofluorescent neurolysosomal deposits of lipopigments. Cerebral and cerebellar atrophy.	(Boehme *et al*. 1971, Noskova *et al*. 2011, Velinov *et al*. 2012, Cadieux-Dion *et al*. 2013, Burgoyne & Morgan 2015)
α-synuclein *(SNCA)*	Parkinson's disease (PD)	168601, 605543	?	Early adulthood to 40s (early onset); 50s or after (late onset)	No	No	No	Parkinsonism (tremor, rigidity, bradykinesia, postural instability)	No	No	Yes	Yes	Presence of Lewy bodies/neurites.	(Deng *et al*. 2018)
	Lewy body dementia (PBD)	127750	?	From 50 years	No	No	No	Parkinsonism	No	No	Yes	Yes	Presence of Lewy bodies/neurites. Dementia	(Whittaker *et al.* 2017, Orme *et al*. 2018)

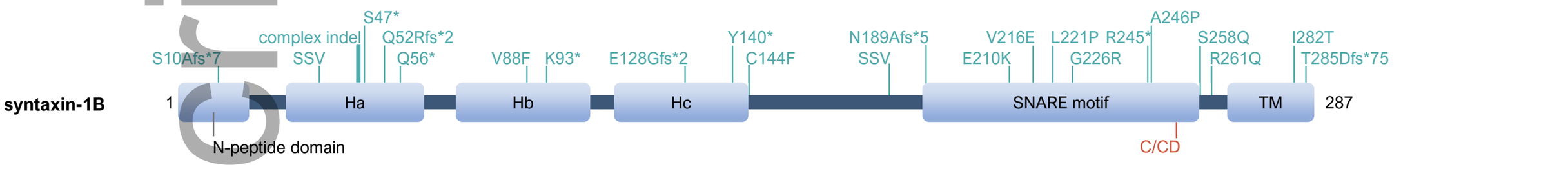
Protein *(GENE)*	Disorder	OMIM#	Cases	Onset	ID	DMD	Seizures	Motor impairments	Muscle weakness	Opth. ab.	Cog. dec.	Neuro-degen.	Other notable features	References
													onset occurs within 1 year of motor symptoms.	
	Multiple system atrophy (MSA)	146500	?	From 30 years	No	No	No	Ataxia, Parkinsonism in some	No	Some	Yes	Yes	Presence of glial cytoplasmic inclusions. Progressive autonomic dysfunction.	(Whittaker et al. 2017)
Tomosyn-2 ***(STXBP5L)***	Fatal infantile-onset neurodegenerative disorder (unnamed)	-	2	Early infancy (3 months of age)	Yes	Yes	Yes	Dyskinesia, dystonia, hypotonia, loss of myotatic reflexes, sensorimotor axonal neuropathy	Yes	Yes	Yes	Yes	Developmental cognitive delay occurs prior to progressive cognitive decline.	(Kumar *et al.* 2015)
PRRT2 ***(PRRT2)***	Paroxysmal kinesigenic dyskinesia (PKD)	128200	>560	1-20 years	No	No	No	Involuntary movement attacks (including chorea and/or dystonia)	No	No	No	No	Attacks generally have a kinesigenic trigger. Sensory aura may precede attacks. Frequency of attacks reduces with age and can completely remit.	(Ebrahimi-Fakhari *et al.* 2018)
	Benign familial infantile seizures (BFIS)	605751	>600	First year of life	No	No	Yes	None	No	No	No	No	Complex partial seizures, with unilateral motor component. Generally resolves by two years of age.	(Ebrahimi-Fakhari et al. 2018)
	PKD with infantile convulsions (PKD/IC)	602066	>210	First year of life	No	No	Yes	Involuntary movement attacks (including chorea and/or dystonia)	No	No	No	No	Individuals exhibit symptoms of both PKD and BFIS.	(Ebrahimi-Fakhari et al. 2018)
Complexin-1 ***(CPLX1)***	Early infantile epileptic encephalopathy 63 (EIEE)	617976	5	Early infancy	Yes	Yes	Yes	Cerebral movement disorder, cerebral palsy, hypotonia	No	No	No	?	Global developmental delay.	(Karaca *et al.* 2015, Redler *et al.* 2017)

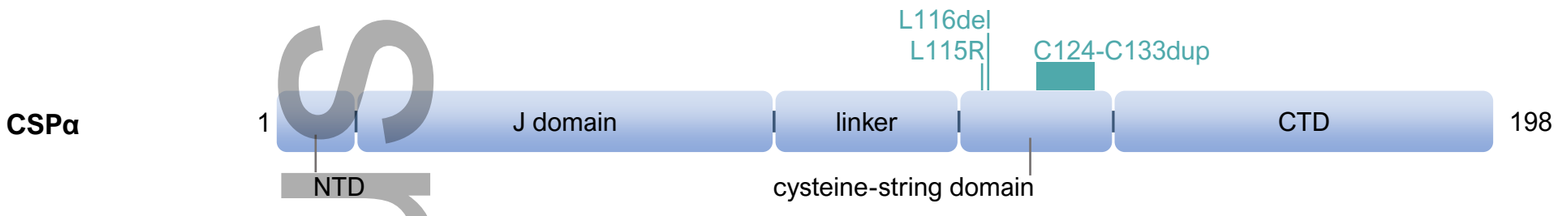
Protein *(GENE)*	Disorder	OMIM#	Cases	Onset	ID	DMD	Seizures	Motor impairments	Muscle weakness	Opth. ab.	Cog. dec.	Neuro-degen.	Other notable features	References
Synaptotagmin-1 ***(SYT1)***	Baker-Gordon syndrome (BAGOS)	618218	11	Birth	Yes	Yes	No	Abnormal involuntary movement, ataxia, choreoathetosis, dystonia, hyperkinesis, infantile hypotonia	No	Yes	No	No	Abnormal behaviour, episodic agitation, stereotypies, abnormal EEG but no overt seizures.	(Baker *et al.* 2018)
Synaptotagmin-2 ***(SYT2)***	Congenital myasthenic syndrome 7 (CMS7)	616040	>20	Birth or childhood	No	Some	No	Dominant: Hyporeflexia, gait abnormalities, distal limb weakness; Recessive: Severe hypotonia, areflexia, severe muscle weakness (limb, axial, facial)	Yes	Some	No	No	Differential presentation for dominant-negative and recessive loss-of-function mutations. Foot deformities (pes cavus, hammer toes) common for dominant mutations.	(Herrmann *et al.* 2014, Montes-Chinea *et al.* 2018, Donkervoort *et al.* 2020, Maselli *et al.* 2020)

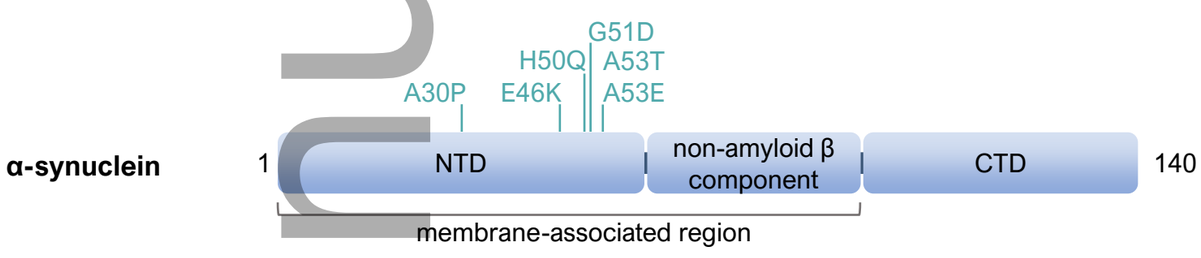


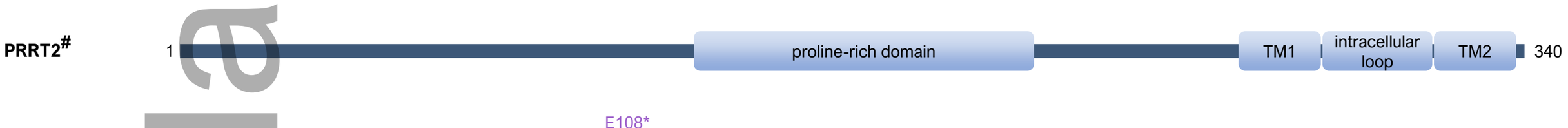


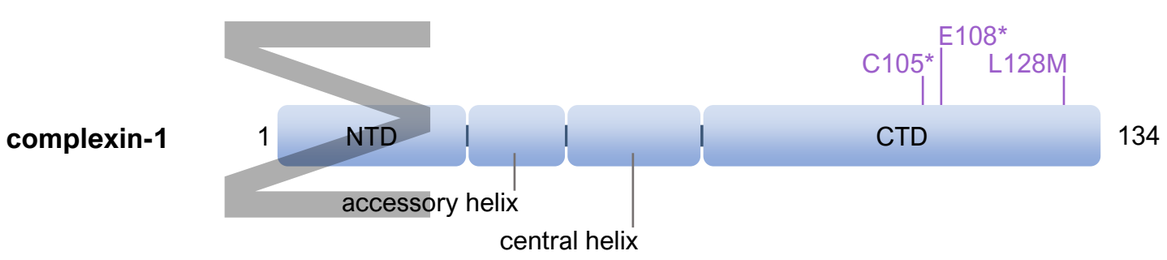


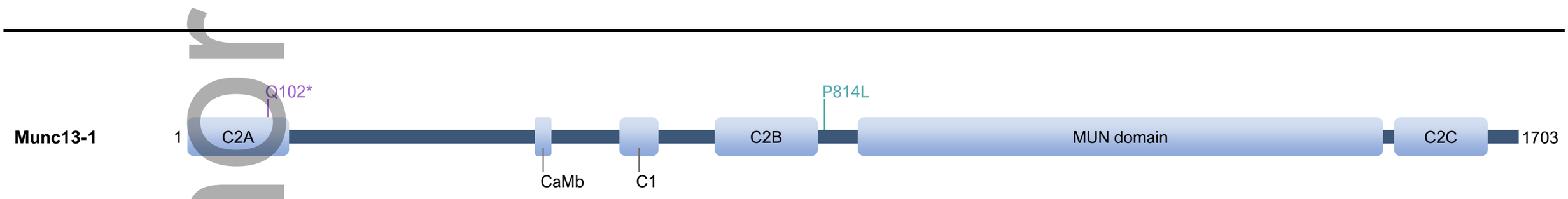


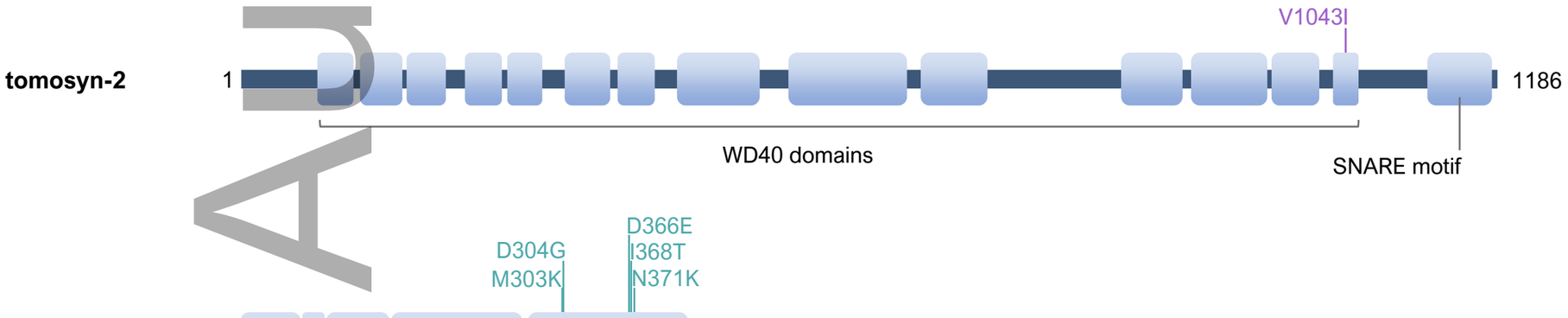


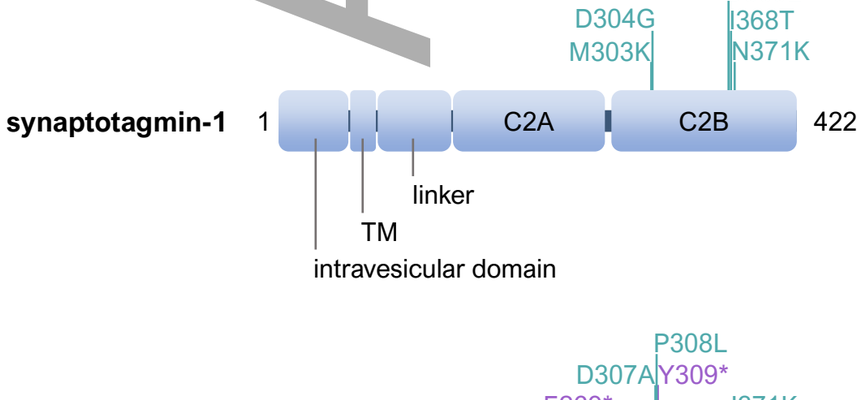


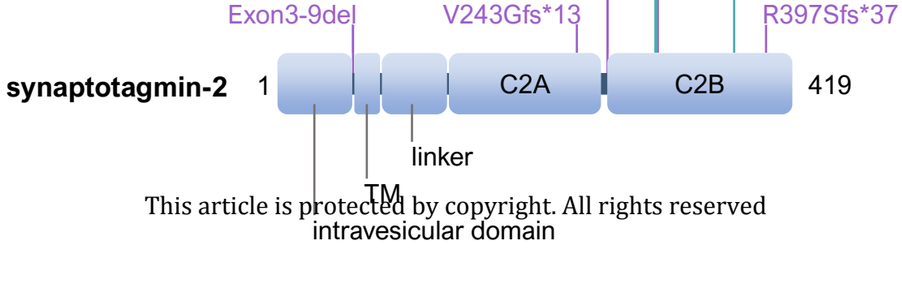


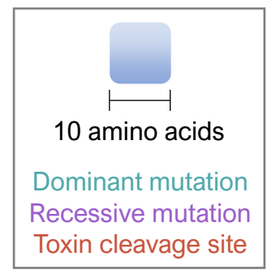


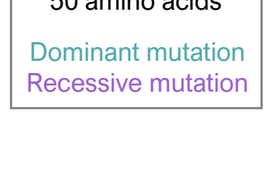


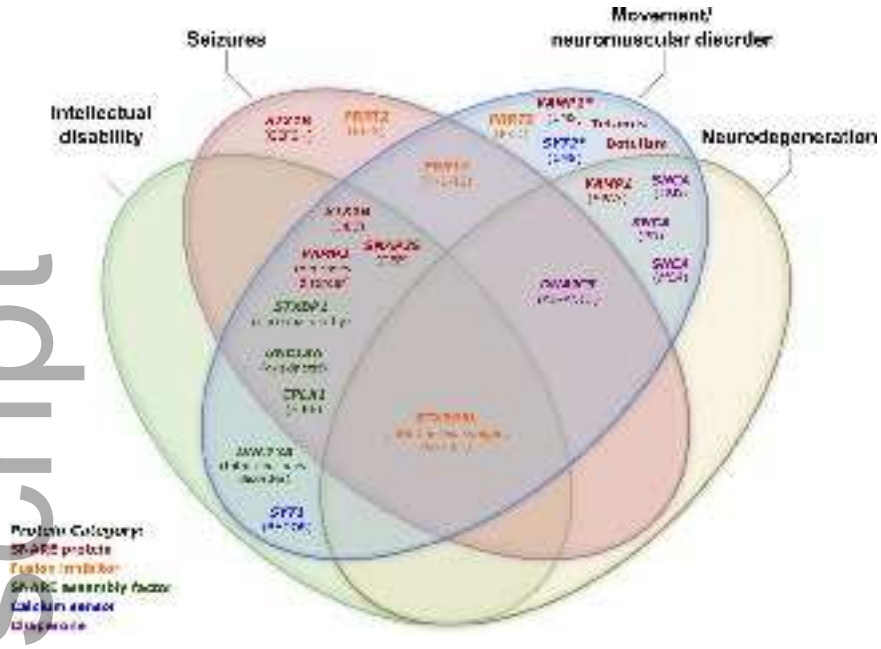












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